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School of Pathology

The effect of chronic preconception paternal alcohol intake on sperm methylation signatures and subsequent gene expression in mouse offspring

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Declaration

I, Jaysen Gregory Knezovich, hereby declare this dissertation to be my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been previously submitted for any degree or examination at this, or any other university.

Signed this 29th day of August 2014

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Sempre.

Abstract

Alcohol is known to be a potent teratogen affecting neuronal development and cellular growth, resulting in a range of clinical manifestations including cognitive and behavioural abnormalities, growth restriction and craniofacial malformations. Much research has focussed on maternal exposure, but few studies have explored the role of preconception alcohol exposure in the father. This study aimed to investigate the effect of chronic preconception alcohol exposure on sperm DNA methylation signatures in male mice, and assess the outcome of their offspring.

Epigenetic mechanisms that regulate gene expression include DNA methylation, histone modifications and non-coding RNAs. Environmental exposures, including alcohol, have been shown to influence these regulatory mechanisms and alter the epigenetic state of the male gamete. Aberrant DNA methylation signatures are transmissible through the paternal germline to subsequent generations, and are associated with abnormal phenotypes in their offspring. However, the causal mechanism of these altered phenotypes has been largely unexplored.

In this study, a mouse model was developed to investigate the impact of chronic paternal preconception alcohol exposure on sperm DNA methylation at CpG islands throughout the genome and locus specific imprinting control regions, and gene expression in the offspring of exposed males. The hypothesis is that chronic alcohol exposure induces DNA methylation changes in the sperm, which are transmitted through the male germline and compromise epigenetic regulation of gene expression in their offspring. This may manifest as phenotypic features similar to those observed in children with foetal alcohol spectrum disorders (FASD). Reduced representation bisulfite sequencing, which enriches for CpG islands, was used to quantify sperm DNA methylation at single-base resolution. Significant alterations in the

sperm methylome were observed, which were associated with genes enriched for biological processes that included central nervous system development, immunity, and heart development – all of which are related to phenotypes observed in FASD. Paternal preconception alcohol exposure was associated with significantly decreased brain and liver weights in 16.5 day old male embryos. The growth-restricted phenotype was associated with dysregulated gene expression profiles in the embryonic brain, liver and placenta. Gene pathway enrichment analysis revealed biological processes related to embryonic development and apoptosis, which are aligned with the observed embryonic growth restriction phenotype. An overlap analysis revealed that 38 genes were both abnormally methylated in the sperm of alcohol exposed sires, and aberrantly expressed in embryonic tissues of their offspring. Overall, the findings of this study provide compelling evidence for alcohol-induced sperm DNA methylation aberrations with associated gene expression and phenotypic alterations in offspring sired by males chronically exposed to alcohol prior to conception. This suggests that epigenetic mutations in the gamete are transmissible to subsequent generations and can alter embryonic gene expression in a tissue-specific manner, and contribute to phenotypes related to the clinical signs observed in children with foetal alcohol spectrum disorders. These data provide further evidence for paternal contributions to disease, and suggest that a more complex public health intervention, including education of prospective fathers, is necessary to reduce the effects of alcohol in societies where there is excessive consumption.

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List of abbreviations

µl	Micro litre
5’	Five prime
5hmC	5-hydroxymethlayed methylcytosine
5mC	5-methylcytosine
ADHD	Attention-deficit hyperactivity disorder
ALD	Alcoholic liver disease
ARBD	Alcohol-related birth defects
ARND	Alcohol-related neurodevelopmental disorder
A^{vy}	Augouti variable yellow
BAC	Blood alcohol concentration
BMP	Bone morphogenic protein
bp	base pair(s)
CAM	Cambridge mouse model
CDC	Centre for Disease Control
CGI	CpG island
CNS	Central nervous system
CpG	Cytosine-phosphate-Guanine dinucleotide
CTCF	CCCTC-binding factor/site/region
CTRL	Control
ddH_2O	Double-distilledwater
DMR	Differentially methylated region
Dnmt	DNA methyltransferase
dpp	Days post coitus
Dpp	Days post-partum
E16.5	Embryonic age of 16.5 days
ESC	Embryonic stem cell(s)
ETOH	Ethanol
FAE	Foetal alcohol effects
FAS	Foetal alcohol syndrome
FASD	Foetal alcohol spectrum disorders
FC	Fold change in gene expression
FMR	Fully methylated region

g	Gram(s)
GO	Gene ontology
GWAS	Genome-wide association study
HED	Heavy episodic drinking
HNPCC	Hereditary nonpolyposis colorectal cancer
ICM	Inner cell mass
ICR	Imprinting Control Region
IFN	Interfernon
IUGR	Intrauterine growth restriction
kb	kilobase(s)
kg	Kilogram(s)
LMR	Low methylation region
MBD	Methyl-CpG binding
MeDIP	Methylated DNA immunoprecipitation
mg	Milligram(s)
miRNA	Micro RNA
ml	Millilitre(s)
mRNA	Messenger RNA
NCD	Non-communicable disease
ncRNA	Non-coding RNA
ng	Nanogram(s)
NO	Nitric oxide
PCA	Principal components analysis
PCD	Programmed cell death
PCR	Polymerase chain reaction
pFAS	Partial FAS
PGC	Primordial germ cell(s)
PTSD	Post-traumatic stress disorder
QC	Quality control
RA	Retinoic acid
RRBS	Reduced Representation Bisulfite Sequencing
SA	South Africa mouse model
siRNA	Short-interfering RNA
Ta^{o}C	Annealing temperature in degrees Celsius

TE	Trophectoderm
TGS	Transcriptional gene silencing
UMR	Unmethylated region
WHO	World Health Organisation
XCI	X-chromosome inactivation

Chapter 1

Introduction

1.1. Diseases of lifestyle

According to the World Economic Forum's 2009 report, non-communicable diseases (NCDs) are among the most severe threats to global economic development. NCDs are said to be potentially more detrimental than fiscal crises, natural disasters, or pandemic influenza (as stated in Narayan et al., 2010). The World Health Organisation (WHO, 2010) reported that in 2008, NCDs accounted for approximately 63% of global mortalities – equating to 36 million deaths. Of these deaths, 29% were premature (before the age of 60 years old). The vast majority of NCD-related mortalities are attributed to cardiovascular diseases, cancers, diabetes and chronic lung diseases. Four behavioural risk factors associated with economic transition, rapid urbanisation, and modern lifestyles underlie the cause of NCDs. These include unhealthy diet, tobacco use, insufficient physical activity, and alcohol abuse. Respectively, these factors account for the deaths of approximately 12.9 million (which encompasses complications associated with raised blood pressure, obesity, and raised cholesterol), 6 million, 3.2 million, and 2.3 million people per year (Casswell et al., 2011; WHO, 2010).

Globally, NCD-related deaths are predicted to increase by 15% between 2010 and 2020 (WHO, 2010). The gravity of this escalation is realised by the fact that NCDs are expected to

supersede communicable, maternal, perinatal, and nutritional diseases combined, as the leading cause of death by 2030. The WHO foresees that the greatest increases will be in the Africa, South-East Asia and Eastern Mediterranean regions, where they will increase by over 20%. The African region alone is expected to suffer 3.9 million NCD-related deaths by 2020. This foreseeable rise has been attributed to the fact that people in developing countries are being increasingly exposed to, and consequently increasingly eating foods with higher levels of total energy and are being targeted by marketing for tobacco, junk food, and alcohol.

1.1.1. Alcohol misuse – a cause for concern

The average global annual alcohol consumption in 2005 was 6.13 litres of pure alcohol per person (≥15 years of age) (WHO, 2011). However, significant variation exists in adult per capita consumption (Figure 1). In addition to Argentina, Australia and New Zealand, the highest consumption levels are generally observed in developed countries in the northern hemisphere. Medium consumption levels are found in North America, most of South America, and southern Africa (with Namibia and South Africa having the highest levels). As would be expected, the lowest consumption levels are found in countries that abstain from alcohol use for religious reasons, which include North and sub-Saharan Africa, the Eastern Mediterranean region, and southern Asia (WHO, 2011).

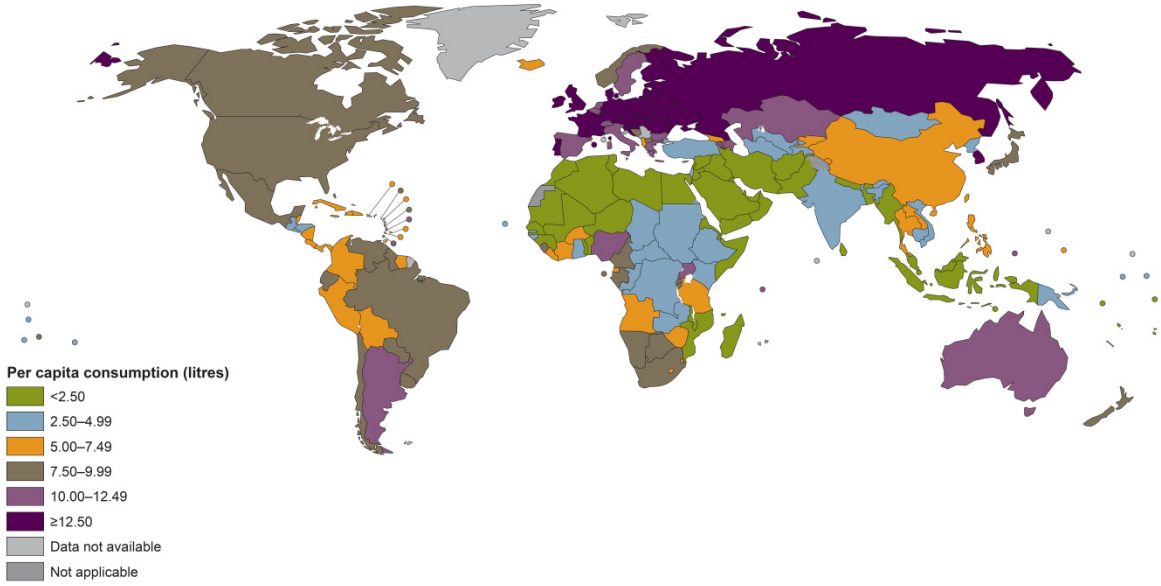


Figure 1: Total adult (≥15 years of age) per capita consumption, in litres of pure alcohol, 2005 (WHO, 2011)

Drinking behaviour also differs considerably throughout the world (Figure 2). The frequency and quantity in which people consume alcohol can be scored on a 5-point scale (WHO, 2011), where 1 represents the least risky (lowest frequency and quantity) pattern of drinking behaviour, and 5 being the most risky (highest frequency and quantity). Scores are calculated using several factors that include frequency of drinking, intake with food, and the quantity of alcohol consumed per occasion. High drinking pattern scores prevail in Kazakhstan, Mexico, the Russian Federation, Ukraine and South Africa. Heavy episodic drinking is one of the most important indicators for acute consequences of alcohol use, such as injuries. Heavy episodic drinking is relatively high in many countries with middle to high per capita consumption, such as in Brazil and South Africa. Intermediate to low scores are noted in the Americas, Australia, and most of Asia and Africa, while the United Kingdom and Europe have the least risky drinking behaviour (WHO, 2011).

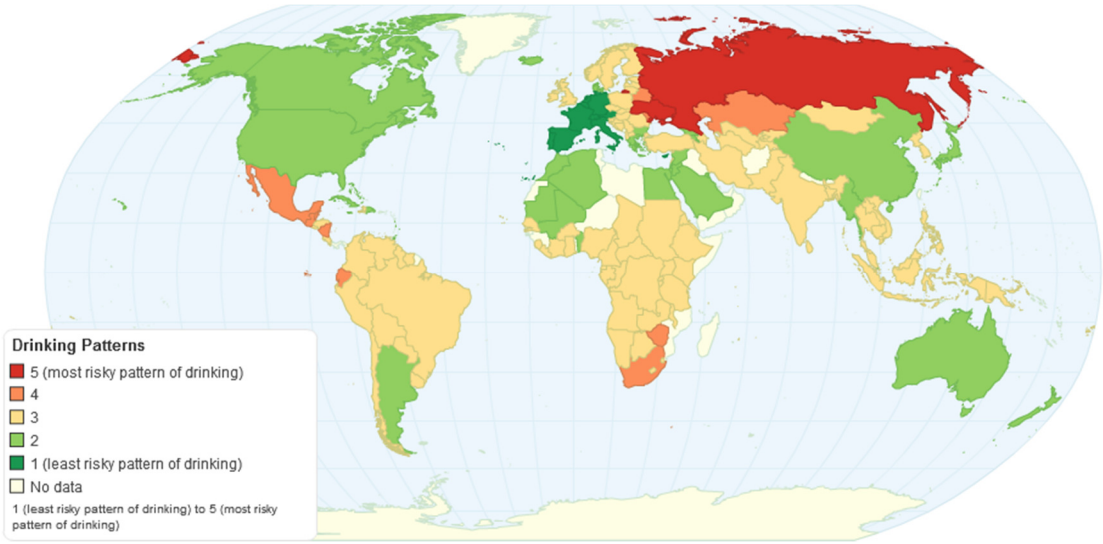


Figure 2: Worldwide Patterns of Drinking Score in 2005 (ChartsBin statistics collector team 2011, Worldwide Alcohol Drinking Patterns, ChartsBin.com, viewed 22 April 2012, www.chartsbin.com)

Findings by the WHO suggest that average consumption levels appear to be relatively stable globally (as observed from 2001 to 2005), with regional increases in parts of South America, Africa, northern Europe and South-East Asia. These increases are of particular concern in countries that already exhibit high consumption levels and/or risky drinking behaviour such as Finland, Kazakhstan, the Russian Federation and Zimbabwe.

Almost 4% of all deaths worldwide are attributed to alcohol (WHO, 2011). This is greater than the deaths caused by HIV/AIDS or tuberculosis. Alcohol consumption is the world's third largest risk factor for disease and disability, and is the greatest risk in middle-income countries. Alcohol is a causal factor in 60 types of disease and injury, and a component cause in 200 others. The consumption of alcohol is estimated to be the causal factor of nearly 50% of liver cirrhosis cases, epilepsy, poisonings and several types of cancer. Injuries caused by alcohol misuse include accidents associated with drink-driving, and its teratogenic effect on foetal development. Foetal alcohol spectrum disorders (FASD) develop as a consequence of maternal alcohol consumption during pregnancy, with foetal alcohol syndrome (FAS) being

the most severe of these. FASD is associated with life-long mental, physical and behavioural disabilities. Globally, prevalence of FAS ranges between 0.5-2.0 per 1000 live births (May and Gossage, 2001). The highest rate of FAS occurs in mixed ancestry communities in the Western Cape of South Africa, which range between 68.0 and 89.2 per 1000 children of school-going age (May et al., 2007).

Alcohol is also associated with many serious social issues, including violence, child neglect and abuse. Lastly, excessive alcohol consumption has significant economic cost due to loss of productivity, and costs associated with healthcare and criminal justice. It was estimated that the United States lost $223.5 billion in 2006 as a direct cause of alcohol misuse (Bouchery et al., 2011). Furthermore, the cost of medical care for a child with foetal alcohol syndrome in the USA is estimated to be US$2 million per individual (Lupton et al., 2004).

Overall, these findings demonstrate a global trend of relatively high per capita consumption of alcohol, which is in most cases, associated with risky drinking behaviour. The fact that these trends are on the rise, in conjunction with alcohol's association with a number of diseases, demonstrates that alcohol abuse is likely to be a significant contributor to the concerning global increase in NCDs. The aetiology of NCDs commonly involves the interaction of environmental exposures with the genome, where exposures can influence genetic regulation, and phenotype outcome.

1.1.2. A case of missing heritability

Genome-wide association studies (GWAS) have been used to study the heritability of complex traits, such as height and diseases, with little success. More than 1,200 loci containing genetic variants that are associated with approximately 165 common diseases and traits have been identified using GWAS (Zuk et al., 2012). However, despite the

association of these variants with phenotype outcome, they appear to explain only a very small proportion of the heritability and variation in NCD risk within a given population (Maher, 2008; Manolio et al., 2009). At best, GWAS explain about 20 – 30% of trait heritability (Wallace, 2010), and hence, the majority of heritability remains unexplained.

Although there is still much debate over the whereabouts of the "missing heritability" of disease – which may be attributed to rare genetic variants with strong effects, the effects of copy-number variations, as well as gene-gene and gene-environment interactions (as stated in Gluckman et al., 2010) – links between prenatal growth and the later risk of NCDs are thought to reflect variations in the quality of the intra-uterine environment (as reviewed in Gluckman et al., 2008; Hanson and Godfrey, 2008). As well as the limiting effects of small uterine size, constrained growth may reflect other aspects of the intra-uterine environment such as nutrition, oxygen supply and hormonal exposure (Hanson et al., 2011). Lower birth weight within the normal range for a particular population is associated with an increased risk in later life of cardiovascular disease (Fall et al., 1995; Rich-Edwards et al., 1997) and the development of metabolic syndrome, which is associated with hypertension (Moore et al., 1996), insulin resistance, type II diabetes (Barker et al., 1993), dyslipidaemia and obesity (Nobili et al., 2008) Similarly, at the highest birth weight range, the risk of disease, including breast cancer (Michels and Xue, 2006), testicular cancer (Michos et al., 2007), and childhood leukaemia (Hjalgrim et al., 2003) increases, resulting in a U- or J-shaped relationship between birth weight and later disease risk (Lillycrop and Burdge, 2011).

In addition to adulthood disease, low birth weight has been shown to be associated with a diversity of cognitive disorders in later life (as reviewed in Harris and Seckl, 2011). Low birth weight has been linked to schizophrenia, attention deficit/hyperactivity disorder (ADHD), antisocial behaviour, increased vulnerability to post-traumatic stress disorder (PTSD), anxiety disorders, learning difficulties and depression

1.1.3. Developmental origins of non-communicable disease

It has been known for almost 100 years that the environment in which the embryo and foetus develops can induce variations in the phenotype of the offspring without changing the genome (Lillycrop and Burdge, 2011). Some of the earliest epidemiological evidence that early life events were associated with risk of disease came about in the 1930s, where it was suggested that childhood conditions influenced later mortality (Kermack et al., 1934). In the early 1970s, a series of papers by the East German endocrinologist, Günter Dörner, and his group proposed that the conditions before and soon after birth were related to later risks of arteriosclerosis and obesity, and that gestational diabetes presented a risk for subsequent diabetes mellitus in adult life (Gluckman et al., 2010)

According to Barker's foetal origins of adult disease hypothesis, perturbations in the gestational milieu influence the development of diseases later in life (Barker, 1995). Foetal development is a critical period in which genetic and cellular systems are programmed to form a functional organism, and is particularly sensitive to the maternal environment. Perturbations to this environment may significantly impact on cellular proliferation and differentiation that can abrogate the developmental pathway of normal tissues and organs. Disruptions to normal foetal development include nutritional factors, psychological or physiological stressors, and imbalances in the normal endocrine signalling between the mother and the foetus (Langley-Evans and McMullen, 2010).

"Programming" describes the process by which a stimulus or insult applied at a critical period of foetal development results in long-term effects on the structure or function of an organism (Lucas, 1991). This critical period will vary for different organ systems, and consequently developmental programming could occur at various time points during the development of an organism, including conception, foetal life and infancy (Ozanne and

Constancia, 2007). The theory of developmental programming (in terms of *in utero* development) relies on the plasticity of the foetus. That is, the developmental trajectory of the foetus is dynamic. The processes of developmental plasticity are invoked over the normative range of environmental experiences, enabling an organism to modulate its phenotypic development and life course in potentially adaptive ways (Low et al., 2011) (Figure 3). According to the theory, the developing foetus anticipates, and adaptively responds to, external environmental challenges in order to better prepare itself for the environment into which it will be born (Langley-Evans, 2006; Lucas, 1998). This provides a mechanism by which permanent characteristics of an individual are founded based on environmental conditions early in life, which modify the developmental trajectory of that individual. For example, it has been shown that stress during pregnancy in rats influences foetal development, and offspring exhibit higher levels of anxiety (Maccari et al., 2003). According to the theory, in this instance, the stress-phenotype is a risk-avoidance adaption to avoid the predators that initially stressed the mother during pregnancy.

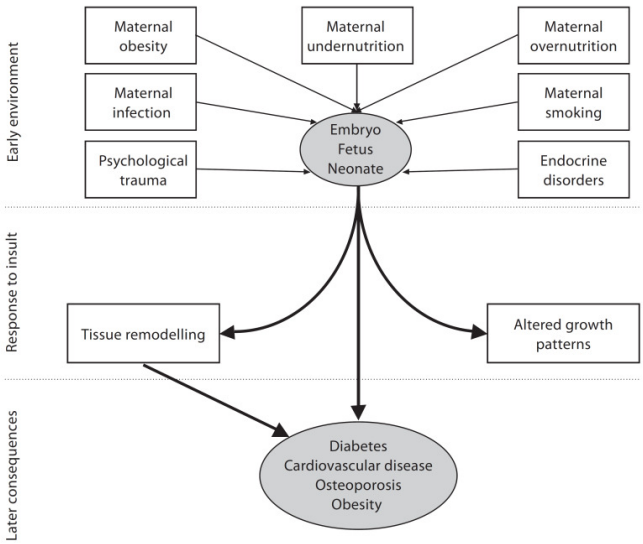


Figure 3: The foetal origins of adult disease hypothesis. (Langley-Evans and McMullen, 2010)

There are three compelling examples of environmentally-induced adaptive responses that phenotypically enhance the fitness the foetus for its pending postnatal environment. When challenged by overcrowding, the desert locust (*Scistocerca gregaria*) induces the production of offspring that are gregarious, diurnal, and migratory in contrast to the nocturnal, sedentary forms that are produced under low population density (Pener and Yerushalmi, 1998). The offspring of *Daphnia* are born with a defensive "helmet" structure if their mother has been exposed to chemicals produced by predators (Laforsch and Tollrian, 2004). The duration of daylight to which meadow voles (*Microtus pennsylvanious*) are exposed during pregnancy determines coat thickness in the offspring in anticipation of winter or spring temperatures (Lee and Zucker, 1988).

Some adaptive responses are not induced by environmental challenges, but rather by deliberate environmental cues. Female honey bees (*Apis mellifera*) are genetic clones of each other. However, queens are distinct from workers in their morphology, capacity to reproduce, behaviour, and longevity. The difference between the queen and worker castes lies in the exposure of the genetically identical larvae to royal jelly (an as yet incompletely defined mixture of proteins, amino acids, vitamins, lipids, and other nutrients) (Maleszka, 2008). All larvae are fed for the first three days after hatching, but only those destined to become queens are fed royal jelly thereafter (Burdge and Lillycrop, 2010).

Adaptations which alter developmental trajectory often manifest as irreversible changes to tissue structure and function. This however, can modulate physiological function and disease susceptibility in later life. As in other species, developmental plasticity attempts to "tune" gene expression to produce a phenotype best suited to the predicted later (adult) environment. When the resulting phenotype is matched to its environment, the organism will remain healthy. However, individuals who have anticipated and adapted to one environment in utero, are exposed to another, perhaps ill-suited environment, a mismatch

occurs, potentially putting the individual at risk (Bateson et al., 2004). When such a mismatch occurs, the individual's ability to respond to environmental challenges may be inadequate and risk of disease increases. Thus, the degree of the mismatch determines the individual's susceptibility to chronic disease (Godfrey et al., 2007). For example, in humans, weight gain up to 25 weeks gestation is primarily due to linear growth. Accumulation of body fat is initiated at about 25 weeks of gestation and approximately 40% of the variation in birth weight reflects differences in the magnitude of fat deposition. Thus, infants born with a lower birth weight are likely to have a reduced fat mass. Small babies who undergo early catch-up growth that is characterised by a greater accumulation of fat mass relative to lean body mass, have an increased risk of becoming obese in later life compared with those born at higher birth weights (Lillycrop and Burdge, 2011).

Persistent changes in tissue structure and physiology during foetal development play an important role in determining the risk of developing NCDs. Birth weight is seen as a readily measurable proxy for the overall maternal, environmental and placental factors that influence the efficiency of nutrient transfer to the foetus, and thus foetal growth. It is generally posited that insults to the *in utero* environment manifest as low birth weight. The first evidence supporting early nutrition as a stimulus for developmental programming comes directly from epidemiological studies in human populations, particularly from the Dutch Winter Famine during World War II (Stein and Susser). During the Dutch famine, women who experienced dietary deficiencies during late gestation gave birth to offspring with reduced birth size, and these infants were found to have an increased risk of developing glucose intolerance and obesity in adult life. Animal studies supporting this have shown that foetal growth restriction in the mouse resulting from maternal protein restriction followed by postnatal catch-up growth and diet-induced adult obesity resulted in a reduction in longevity (Ozanne and Hales, 2004). Both human and animal studies have demonstrated an

association between low birth weight and the risk of developing type II diabetes (Pilgaard et al., 2010), obesity (Taylor and Poston, 2007), cardiovascular disease (Rich-Edwards et al., 1997), osteoporosis (Antoniades et al., 2003), allergic disorders, some cancers (Spector et al., 2009), elevated blood pressure, elevated fat-free body mass in adults (as reviewed in Kiani and Nielsen, 2011), as well as cognitive and other mood disorders (Gale and Martyn, 2004; Scott et al., 2012)

1.1.4. Gene-environment interactions

Gene–environment interactions are central to the concept of programming. Cells exposed to an insult must pass the effects of the exposure to their descendants, in order for changes in gene expression to be maintained through subsequent cellular divisions. A mechanism that allows for the stable propagation of gene activity-states from one generation of cells to the next is thereby required (Ozanne and Constancia, 2007). Developmental plasticity occurs when environmental influences affect cellular pathways during gestation, enabling a single genotype to produce a broad range of adult phenotypes (Dolinoy et al., 2007)

Cells specific to a particular tissue type have identical genetic information to those of any other tissue. However, it is the uniquely orchestrated expression of a combination of genes within a given tissue that gives rise to its distinct form and function. The tissue-specific gene expression profiles across various cell types are achieved through the exertion of external biological forces that control gene expression. In 1942, Conrad Waddington (as recently re-published in Waddington, 2011) coined the term “epigenetics” to describe “the science concerned with the causal analysis of development”. His idea was conceptualised in the form of a “genetic landscape” (Figure 4), whereby his theory of development follows the passage

of a fertilized egg or cell down a “landscape”. The unique path that each cell follows through the landscapes canals will ultimately determine its developmental state – a process known as “canalization”. The term “epigenetics” has subsequently been redefined by Robin Holliday as the “unfolding of the genetic program for development” (Holliday, 2006), and more recently by Adrian Bird as the “structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” (Bird, 2007). Ultimately, epigenetics encapsulates factors independent of the DNA sequence that regulate gene expression.

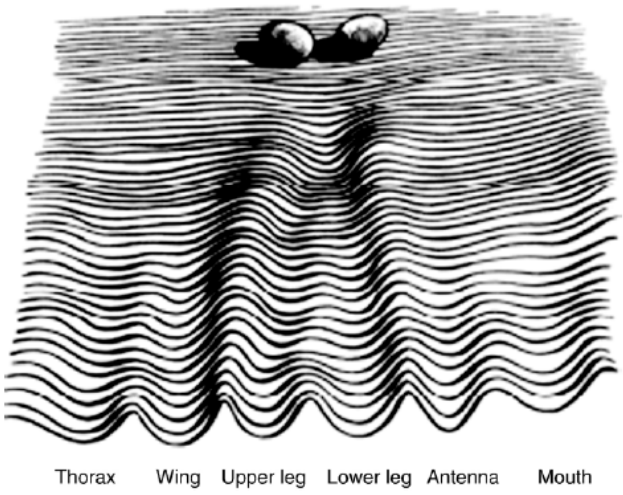


Figure 4: Conrad Waddington's epigenetic landscape. (Waddington, 1975)

Developmental programming is fundamentally based on epigenetic regulation of gene expression. Epigenetic modifications are modulated by the environment and act as reversible switches of gene expression that can lock genes in active or repressed states. Epigenetics might thus provide a link between a suboptimal early environment and long-term effects on key components of metabolic pathways (Ozanne and Constancia, 2007), which consequently be responsible for development of disease in adult life.

1.2. Epigenetic regulatory mechanisms

There are two epigenetic mechanisms by which tissue-specific gene expression patterns are established and maintained. These include chromatin regulation, involving DNA methylation and histone modifications, and RNA interactions (Figure 5).

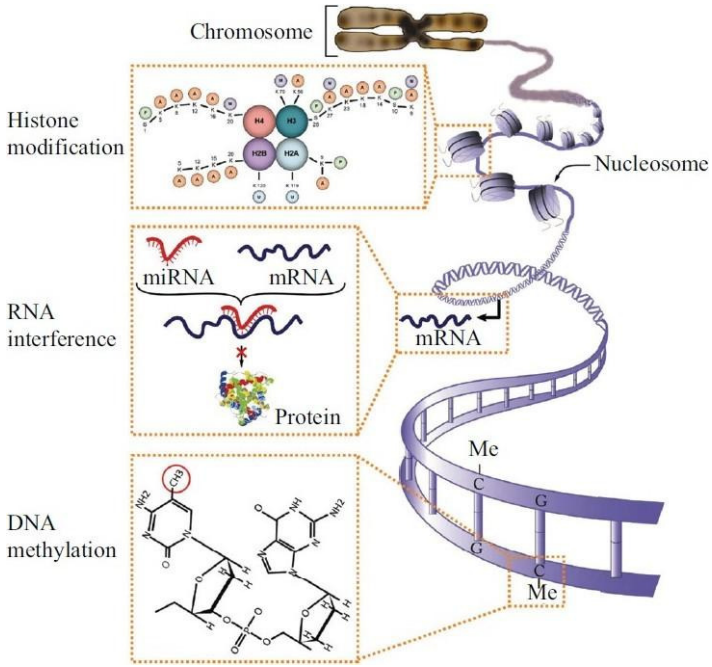


Figure 5: Overview of epigenetic mechanisms. (Kim et al., 2011)

1.2.1. Chromatin conformation and gene regulation

Eukaryotic genomes are organised into chromatin – a DNA-protein complex that packages the DNA into a highly compact form. The functional unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped around an octamer of histone proteins (as described in Ooi and Henikoff, 2007). The octamer core consists of two copies of each of the

histones, H2A, H2B, H3 and H4, and each nucleosome is connected by the linker histone, H1 (Campos and Reinberg, 2009). The DNA and protein components of the nucleosome are subject to a variety of modifications that influence chromatin accessibility. In addition to these chromatin modifications, further remodelling of the chromatin into a higher order structure, ultimately regulates gene activity in a precise temporal and spatial manner.

1.2.1.1. Histone modifications

Each core histone has an unstructured N-terminal tail that protrudes from the nucleosome, and is subject to posttranslational modifications that include methylation, acetylation, phosphorylation, sumoylation, ADP-ribosylation, deamination, proline isomerisation and ubiquitylation at specified residues (as revewied in Kouzarides, 2007). Importantly, these modifications are dynamic – being reversible by enzymes that direct their removal. Histone modifications essentially function to help partition the genome into two functional domains: the transcriptionally active euchromatin, and the transcriptionally inactive heterochromatin. To achieve this, modifications are coordinated in such a way that either opens the chromatin, making it accessible to cellular proteins (euchromatic regions); or condenses the chromatin, rendering inaccessible to protein interactions (heterochromatin). By disrupting the interaction of histone proteins within adjacent nucleosomes, higher order chromatin structure is destabilised and therefore more accessible to transcription factors. Acetylation neutralises the positively charged lysine residue, which reduces its affinity for DNA and allows the nucleosomes to unfold, and the accessibility of transcription factors (Lee et al., 1993) .

In addition to their influence on chromatin structure, histone modifications are able to recruit proteins that bind through specific domains. Recruitment in this manner tethers

various enzymes to the chromatin, including deacetylases, demethylases, deacetylases and ATPases which act on proximate histone residues and influence chromatin structure (as reviewed in Bannister and Kouzarides, 2011; Kouzarides, 2007). Conversely, histone modifications are also able to prevent the binding of proteins to certain chromatin regions (Margueron et al., 2005).

As described by Kouzarides (2007), the abundance of modifications that co-exist on a histone tail and their proximity to one another, opens the likelihood of "crosstalk" between them. Several lines of evidence suggest that histone modifications can act synergistically with, or antagonistically to, one another, which can significantly influence chromatin structure and modulate gene activity. Firstly, protein binding can be abrogated by the presence of a neighbouring modification. This has been demonstrated by evidence that heterochromatin protein 1 bound to a trimethylated lysine 9 residue, is ejected by the presence of a phosphorylated serine 10 (Fischle et al., 2005). Secondly, the catalytic activity of proteins that interact with histones can be affected by modifications. Nelson et al. (2006) showed that the catalytic ability of yeast proline isomerase FPR4 to interact with histone tails to establish a chromatin state that inhibits K36 methylation, is abolished during active transcription where Set2 methylates H3K36. Finally, there is evidence that the presence of an adjacent modification can enhance the recognition of a substrate by its enzyme (Clements et al., 2003).

Chromatin is further characterised by various combinations of core histone variants, the spacing between nucleosomes (nucleosome occupancy) and the position of each nucleosome within the nucleus (nuclear architecture). Modifications to the variety of histones that constitute chromatin, together with their position within, and influence on, genomic structure, have profound influence on genetic regulation.

1.2.1.2. DNA modifications

As early as 1975, two independent research groups put forward the suggestion that DNA methylation was responsible for the stable expression of genes throughout subsequent meiotic cell divisions (Holliday and Pugh, 1975; Riggs, 1975). Today it is known that a proportion of genomic cytosine residues are covalently modified after translation by the attachment of a methyl group to position 5 on the cytosine ring. In eukaryotes, such methylated cytosines are usually found where the cytosine is adjacent to a guanine residue, i.e. in a cytosine–phosphate–guanine (CpG) dinucleotide. Each of these CpG dinucleotides designate potential methylation sites (Robertson, 2005).

DNA methyltransferases (Dnmts) are the only known enzymes that catalyse the methylation of cytosine nucleotides in mammals, and are critical modulators of foetal development (Li et al., 1992). Dnmts make use of the methionine produced via the folate pathway for DNA methylation reactions, which involve the addition of a methyl group to the 5' carbon of a cytosine nucleotide (Rodenhiser and Mann, 2006). To date, there have been three active Dnmts classified in mammals, namely, Dnmt1, Dnmt3a and Dnmt3b. Dnmt2 has been identified as a candidate protein, but has not been shown to be catalytically active (Jeltsch, 2002). Dnmt3a and Dnmt3b contain a domain called PWWP that occurs in proteins known to play a role in cell growth and differentiation (Jeltsch, 2002). These two Dnmts have been highlighted as the *de novo* methyltransferases, and are vital components for establishing methylation patterns. Dnmt1 has been implicated in the maintenance of DNA methylation during DNA replication, ensuring the propagation of specific methylation patterns after each round of cell division (Leonhardt et al., 1992). DNA methylation is the only epigenetic modification that is stably transmitted across mitotic divisions (Margueron and Reinberg, 2010), passing on an epigenetic memory which is crucial for the differentiation state of the

daughter cell (Bird, 2002; Ng and Gurdon, 2008) and subsequent regulation of gene expression.

In approximately half the genes contained within the mammalian genome, unmethylated CpGs are commonly found clustered at the 5' ends of genes, primarily in promoter regions and first exon regions (Jones and Takai, 2001). These CpG dense regions are known as CpG islands (Canani et al., 2011). CpG islands are genomic regions of approximately 1kb in length which contain a CG content of 50% and an observed-to-expected CpG ratio of 60% or greater (Takai and Jones, 2002). Approximately 60% – 90% of the cytosines in these islands are methylated in mammals, accounting for about 3% - 8% of the total number of cytosine residues (Jeltsch, 2002). In mammals, there are approximately 30 000 CpG islands.

Low-methylated regions are associated with regulatory regions and genes important in development

A study by Christensen et al. (Christensen et al., 2009) analysed 217 non-pathologic human tissues from 10 anatomic sites at 1,413 autosomal CpG loci associated with 773 genes to investigate tissue-specific differences in DNA methylation in an attempt to investigate how aging and environmental exposures contribute to normal variation in DNA methylation. They observed highly significant CpG island-dependent correlations between age and DNA methylation in solid tissues, where loci in CpG islands were associated with a gain in methylation, while loci not contained within CpG islands were associated with a loss of methylation. Manikkam et al. (2012) assessed the transgenerational effects of environmental compounds (plastics, dioxin and jet fuel) on methylated regions of rat sperm DNA. This study employed methylated DNA immunoprecipitation (MeDIP) to assess the DNA methylation status of 15,287 promoters. These authors demonstrated regions of differential

methylation (DMRs) of promoters dispersed across the sperm epigenome. These DMRs were associated with several genomic features, including low density CpG content. These authors defined "low" CpG content as an average CpG density of ~8 CpG sites per 100bp. Similar to this finding, Brinkman et al. (Brinkman et al., 2010) observed that low CpG density (0.05 CpG sites per bp or 5 CpG sites per 100bp) is associated with enhancers that may have critical roles in development. Thus, both promoter and enhancer CpG islands contain low CpG density. In an attempt to determine the effect of dietary folate deficiency on DNA methylation, Okoji et al. (2002) fed adult C57BL/6J mice a diet deficient in methionine, choline and folate and supplemented with arsenic (which exacerbates DNA hypomethylation, as arsenic methylation consumes both SAM and GSH – which are required for DNA methylation synthesis). These authors observed dose-dependent DNA hypomethylation, which was associated with a low CpG-density region upstream of the *Ha-ras* gene (in contrast to a high CpG-density region that did not show evidence of hypomethylation). Together, these findings demonstrate that low CpG density regions (~5-8 CpG sites per 100bp or 5-8%) are generally associated with developmentally important enhancers, and appear to be more sensitive to the environment than regions of high CpG density.

CpG density has been associated with particular DNA methylation profiles. DNA regions with both low and high CpG density are generally associated with hypermethylation (Brinkman et al., 2010). Furthermore, regions of low CpG density are highly enriched (89% occurrence) in intergenic, intronic and exonic regions, while regions of intermediate (medium) CpG density are moderately enriched (59% occurrence) in these regions, and high CpG density are enriched at a much lower level (39% occurrence) in these regions. CpG islands and CpG island-containing promoters are increasingly associated with regions of increasing CpG density (15% at low CpG density regions, 40% at medium CpG density regions, and 59% in

high CpG density regions). Further to this, Meissner et al. (2005) observed that regions of high CpG density (>7% over 300bp) tend to be unmethylated, while CpGs contained within low CpG density regions (<5%) tend to be methylated. In addition, their study observed that high CpG density promoters are associated with ubiquitous house-keeping genes and highly regulated key developmental genes, while low CpG density promoters are associated with tissue specific genes.

Aligned with observations mentioned above, genomic views not only reveal that the epigenome is partitioned into distinct genomic states (in terms of CpG content and distribution), but also into distinct DNA methylation states (in terms of levels of DNA methylation). The traditional dogma advocated that the genome existed in essentially a bimodal state: most CpG sites are heavily methylated (70-80%), while CpG dense regions (CpG islands) are largely devoid of DNA methylation (~10%). New insights into the distribution and distinct profiles of DNA methylation within the genome have revealed distinct epigenetic states that are associated with genomic context, cell type or disease. While the existence of a largely highly methylated genome (~80-90% of CpGs with >50% methylation, referred to as fully methylated regions (FMRs) has been confirmed, the remaining portion of the genome has been found to exist in two distinct methylation states: one with <10% methylation (unmethylated regions (UMRs) and another with 10-50% methylation (low methylated regions (LMRs) (Szulwach and Jin). UMRs expectedly occur predominantly at unmethylated CpG islands corresponding with transcription start sites. LMRs, however, occur primarily distal to transcription start sites, and a lower CpG density, and coincide with promoter distal gene regulatory elements enriched for transcription factor binding sites (Burger et al., 2013; Stadler et al., 2011). Furthermore, LMRs and UMRs appear to be distinct in CpG content that is inversely correlated with DNA methylation (Szulwach and Jin). LMRs and UMRs have been found to be conserved in human embryonic stem cells,

where the bulk of DNA methylation dynamics during ES cell differentiation occur within these two regions (and more frequently at LMRs) and are indicative of cell type-specific DNA methylation states (Gifford et al., 2013; Xie et al., 2013) These observations may be expected, as LMRs correspond to promoter distal gene regulatory elements, such as enhancers, and histone modifications at enhancers reflect cell type specificity more than modifications at promoters and CpG islands (Heintzman et al., 2007). Subsequent studies have revealed that regions of the genome that exhibit highly dynamic DNA methylation programming (Burger et al., 2013), occur within a relatively small proportion of the genome, which correspond to promoter distal gene regulatory elements in the mammalian brain and a diversity of human cells and tissue types (Lister et al., 2009; Ziller et al., 2013). Studies have shown evidence that LMRs are associated with the insulator protein, CTCF (Hon et al., 2013), and show a strong presence of 5hmC – the presence of which has also been associated with enhancer regions (Stroud et al., 2011). Burger et al. (2013) further revealed that cell type-specific LMRs are enriched for cell-type specific transcription factor motifs, and constitutive LMRs (LMRs present in at least two other cell type methylomes) are enriched for constitutive transcription factors, including CTCF. From this, it is reasonable to conclude that DNA methylation dynamics and the establishment of particular methylation states during cellular differentiation may be an important developmental mechanism which defines cell type-specific epigenetic codes, which orchestrate cellular phenotypes (Szulwach and Jin, 2014). Heintzman et al (2007) demonstrate that LMRs are likely to act as distal enhancers, as they are shown be enriched for predictive features of enhancers, which include chromatin features such as high H3K4 monomethylation relative to H3K4 trimethylation and the presence of p300 histone acetyltransferase, which are active. From this, they conclude that many LMRs, identified solely by their DNA methylation pattern, represent active regulatory regions.

Genomic regulation by DNA methylation

Genetic regulation through DNA methylation occurs via a number of distinct mechanisms. In the first instance, the advent of cytosine methylation prevents the binding of transcription factors, causing strong and heritable transcriptional inhibition (Stoger et al., 1997), effectively silencing gene expression. Secondly, in some instances, instead of being repulsive, DNA methylation can promote the binding of methyl-CpG binding domain (MBD) proteins and the methyl-CpG-binding protein MeCP2 (Jaenisch and Bird, 2003). Together, these methyl-binding proteins function as transcriptional silencers. The final mechanism through which DNA methylation is able to regulate gene activity is by altering the chromatin structure itself. MBD3 for example, is a component of the nucleosome remodelling and histone deacetylase (NuRD) co-repressor complex. Furthermore, dense intragenic DNA methylation has been shown to initiate the formation of a closed chromatin structure and reduce the elongation activity of polymerase II (Lorincz et al., 2004).

1.2.1.3. RNA interactions

Although approximately half of the human genome is transcribed, only 2% of it is protein coding, accounting for less than half of all transcripts (GENCODE 7 data, June 2012 freeze). Recent studies have challenged the central dogma that RNA is a mere messenger between the DNA blueprint and a functional protein, as the human genome has been shown to generate a plethora of small and long noncoding RNA species that demonstrate complex overlapping patterns of expression and regulation (Ponting et al., 2009).

At present, the are over 22,000 noncoding RNA genes (GENCODE 7 data, June 2012 freeze), which transcribe a diversity of noncoding RNAs, which include microRNA (miRNA), long noncoding RNA (lncRNA), large intergenic non-coding RNA (lincRNA), and small nucleolar

RNA (snoRNA) (as described in Baker, 2011). The manner by which these noncoding RNAs to regulate gene expression is diverse and complex. Some interact directly with protein coding transcripts, while others interact with the proteins themselves. miRNAs, for instance, down-regulate target genes by binding to their transcribed mRNA. This interaction either results in degradation of the mRNA or prevents translation. lncRNAs appear to have many roles in regulating gene expression. These include acting as scaffolds for various protein complexes; and guiding protein complexes to target specific regions in the genome (as described in Baker, 2011). lncRNAs have also been shown to be important in the global localisation of chromatin modifying enzymes, and consequently, regulating chromatin structure across the genome (Koziol and Rinn, 2010; Mattick, 2012; Zhou et al., 2010).

More recently, the ability of noncoding RNAs (specifically, lncRNAs) to regulate the transcriptional activity of genes, through a process known as transcriptional gene silencing (TGS), has been demonstrated (as reviewed in Malecova and Morris, 2010).

1.2.2. The interplay of epigenetic regulators

There is a complex interaction with various epigenetic mechanisms during mammalian development and postnatal life. These highly orchestrated epigenetic interactions ensure cellular integrity, tissue function, and ultimately, organismal vitality.

As mentioned previously, a single genome can be differentially regulated through epigenetic mechanisms to elicit a variety of cellular functions and phenotypes. In this vein, epigenetic regulators coordinate the transition of one cell type into another, through the process of differentiation. The differentiation of embryonic stem cells in the various cell lineages during embryonic development is the quintessential process whereby a population genomically

identical cells, regulated by epigenetic mechanisms, gives rise to functionally diverse cell lineages. Epigenetic modifications subsequently ensure that the daughter cells of a terminally differentiated cell are of the same type. This occurs, for example, in the propagation of tissues. Developmental gene promoters of this nature are regulated by DNA methylation, histone acetylation and histone H3K4 and H3K27 methylation (Youngson and Morris, 2013)

X-chromosome inactivation (XCI) highlights the interaction of noncoding RNAs, DNA methylation and histone modifications to achieve dosage compensation in mammals. XCI is a mechanism employed by species which demonstrate heterogametic sexes, to compensate for gene dosage differences between males and females. In mammalian females (XX), one of the X-chromosomes is randomly inactivated in all somatic cells of the early post-implantation embryo. The process of XCI is directed by the complex interaction of the noncoding RNAs, X-inactivation specific transcript (Xist) and its antisense transcript (Tsix), with the Polycomb repressive complex 2 (PRC2) protein to ultimately silence the randomly selected X chromosome, which becomes epigenetically marked by DNA methylation at promoter regions, and transcriptionally repressive histone modifications (reviewed in Lee, 2011).

The interactions of epigenetic mechanisms govern vital processes that are initiated prenatally and continue into the postnatal life of an organism. It is therefore essential that epigenetic mechanisms are primed prior to fertilisation, to ensure that embryonic development is initiated and finely controlled from the point of conception – the union of gametes.

1.3. Gamete-specific epigenetic characteristics

The fusion of haploid gametes during fertilisation is the foundation of mammalian development. The diploid organism attains its full chromosomal complement through the inheritance of half of its genetic material from each parent. This essentially ensures that offspring have a pair of each chromosome, and thereby of each gene (excluding sex chromosomes). However, simply having the correct number of chromosomes is not compatible with normal embryonic growth. Experiments creating parthenogenic zygotes derived from the fusion of two maternal or paternal pronuclei resulted in failure of the zygote to develop (McGrath and Solter, 1984). These results demonstrated that female and male gametes are functionally different from one another, where both are essential for development. The process of rendering the parental genomes functionally different was subsequently termed “imprinting” (Surani et al., 1984).

Imprinting relies primarily on the establishment of DNA methylation in an allele-specific manner to silence one of the parental genes, while leaving the other unmethylated and therefore, active. This is conditioned during gametogenesis, while the parental genomes are separate, and ensures the reciprocal expression of approximately 100 mammalian imprinted genes.

1.3.1. Genomic imprinting

In the early 1980’s, a puzzling question shrouded the field of developmental biology – why parthenogenetic embryos didn’t develop to term. By activating an ovulated oocyte and repressing either the second meiotic or first mitotic division, researchers were able to successfully restore the diploid state of the oocyte with a full set of chromosomes (Solter, 2006). However, these maternally diploid “parthenogenetic” embryos failed to develop, despite having a normal number of chromosomes. One hypothesis to explain this

phenomenon suggested that the "sperm makes an essential physical (non-genetic) or physiological contribution without which development cannot be completed" (Solter, 2006).

In 1984, McGrath and Solter made use of fertilized zygotes to construct zygotes that contained either two male (androgenones) or two female (gynogenones) pronuclei through pronucleus exchange. This experiment satisfied any possible non-chromosome contributions from the sperm and was therefore expected that both androgenones and gynogenones would develop normally. Surprisingly, this was not the case. In both instances, development failed soon after implantation – the androgenones showed poor embryo proper development, while the gynogenones displayed poor development of the extraembryonic membranes, as was the case with the parthenogenones (McGrath and Solter, 1984). This group therefore proposed that the maternal and paternal genomes are functionally different from one another and that both are essential for development. Furthermore, they suggested that specific genes are inherited in such a way that one form is functional, while the other non-functional, and that this is conditioned during gametogenesis. Surani and colleagues (Surani et al., 1984) called this "imprinting", and this has remained as the term used to refer to the process whereby the two parental alleles are rendered functionally different.

The first three imprinted genes were discovered in 1991 in short succession (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991). Barlow et al. demonstrated that the *insulin-like growth factor-2 receptor* (*Igf2r*) gene is only expressed from the maternal chromosome. The *insulin-like growth factor II* (*Igf2*) gene was then reported by De Chiara et al., who showed that only the paternal copy of this gene was expressed. The third imprinted gene, *H19*, was suspected to be imprinted as it lies in an imprinted locus on chromosome 7, and indeed Bartolomei et al. demonstrated that *H19* was maternally expressed. To date there have been over 80 mammalian imprinted genes identified.

Imprinted genes are critical regulators of embryonic growth and development. The embryos of both mice and humans exhibit a number of imprinted genes, some of which are involved in common regulatory pathways (Feil and Berger, 2007). Chromosomal regions that demonstrated parent-of-origin phenotypic effects when inherited uniparentally, were identified using a mouse model carrying translocation chromosomes (Choi et al., 2005). The phenotypes observed ranged from early embryonic lethality to postnatal effects on growth and development. These phenotypes were attributed to the misexpression of imprinted genes present within the uniparentally inherited or parent-specific duplicated region (Cattanach and Kirk, 1985).

Genes that are imprinted are "tagged" for reduced expression by means of cytosine methylation. Imprinting occurs via allele-specific methylation while the genomes are separated in their respective germ lines and is parent-of-origin specific (Lucifero et al., 2002). This means that only one of the parental alleles is imprinted, while the other remains relatively unmethylated. This results in the reciprocal expression of imprinted genes, that is, if a gene is paternally imprinted, it is maternally expressed and *vice versa*.

Although methylation is possible at any CG dinucleotide, DNA methylation of imprinted regions occurs at CpG islands. The CpG islands of imprinted alleles can occur in what are known as differentially methylated regions (DMRs) (Robertson, 2005). DMRs can be divided into two classes, namely, primary and secondary DMRs (Kobayashi et al., 2006). During either spermatogenesis or oogenesis, primary DMRs acquire gamete-specific methylation and maintain this allelic methylation difference throughout development. Conversely, secondary DMRs establish differential methylation patterns after fertilization. The term imprinting control region (ICR) is given to a primary DMR when the loss of that primary DMR results in the aberrant expression of its associated imprinted genes (Kobayashi et al., 2006). In the hypomethylated state, CpG-islands contained within ICRs act as binding targets for

proteins, which may result in either transcription or repression of the gene (Rodenhiser and Mann, 2006). This form of repression is fairly complex and involves the methylation-dependent binding of CTCF proteins (Burns et al., 2001).

1.3.2. Regulation of imprinted regions by CTCF

The CCCTC-binding factor (CTCF) is a zinc finger protein that is able to specifically bind nucleotide sequences resulting in either the activation or silencing of associated genes. Initially, CTCF was identified as a transcriptional repressor in chickens (Baniahmad et al., 1990). Subsequently, the role of CTCF has been implicated in enhancer-blocking activity of several vertebrate boundary or insulator elements (Bell et al., 1999), where it has been shown to be ubiquitously expressed and highly conserved (Gray and Coates, 2005). The CTCF consensus sequence is able to bind the insulator protein, which isolates downstream enhancers from upstream promoters, creating a boundary element, resulting in transcriptional repression.

The insulating property of CTCF is a vital element by which most imprinted loci define monoallelic expression of imprinted genes in a parent-of-origin specific manner. CTCF binding sites are commonly found in ICRs of imprinted loci and bind the protein in a methylation-dependent manner. That is, in a methylated state, CTCF is unable to bind to its target sequence abrogating its insulator function. Conversely, an unmethylated state allows for CTCF binding, preventing communication between gene promoters and downstream enhancers. It is important to note that CTCF binding is not a simple on/off switch. While the bound state (unmethylated CTCF binding site) of CTCF blocks the interaction of downstream enhancers with the promoter of one gene, it allows the enhancer to interact with the promoter of another gene in the imprinted cluster. The interaction of the enhancer is

reversed in the unbound (methylated) state, i.e. the previously suppressed gene is favourably acted upon, while the corresponding gene in the cluster is now silenced (Figure 6). In this vein, the two genes comprising the imprinted cluster are reciprocally expressed depending on the methylation state of the parental ICR.

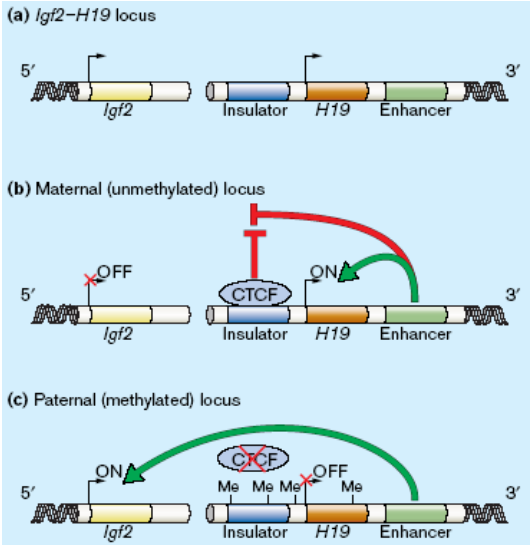


Figure 6: Regulation of imprinted genes by CTCF. (Wolffe, 2000)

The transmission of chromosomes thus not only contributes a genetic DNA component, but also the factors which are able to regulate gene expression. The asymmetry of parental contributions to the zygote further emphasises that epigenetic contributions of each gamete are in part, unique.

1.3.3. Epigenetic contributions from the sperm

The sperm genome contributes both unique genomic material and epigenomic information. The genomic and epigenomic contributions of the sperm ensure correct genome dosage, genome fidelity, appropriate chromatin packaging and convey regulatory elements in the form of regulatory RNAs (as reviewed in Carrell, 2008).

1.3.3.1. DNA methylation signatures

During the pachytene stage of spermatogenesis, during prophase I of meiosis, homologous chromosome recombination takes place and increases genetic diversity of gametes (Topping et al., 2007). A further source of genomic variation contributed by the sperm is in the form of copy number variation (CNV) (McCarroll and Altshuler, 2007). The sperm also contributes unique centrosome machinery to the embryo. The centrosome consists of a pair of centrioles, which are associated with proteins. Together, these elements constitute the microtubule-organising centre of the cell, which is responsible for the proper segregation of chromosomes during cellular division (Manandhar et al., 2005). During spermatogenesis, the distal centriole and some of the associated proteins are eliminated through a process called centrosome reduction. Conversely, in the oocyte, both centrioles are lost but the associated proteins, which are vital for centrosome functioning within the embryo, are retained. During fertilisation, where the sperm penetrates the oocyte, the sperm-derived centriole duplicates and recruits the retained oocyte centrosomal proteins to become a functional embryonic centrosome (Manandhar et al., 2005). Together, the haploid complement of chromosomes, genomic variation and cellular components contained within the sperm, contribute uniquely to genomic dosage of the embryo.

As the findings by McGrath and Solter (1984) alluded to, the haploid paternal sperm genome contributes more than just genomic material. As described previously, parent-of-origin imprinting occurs in the gametes, which ensures the reciprocal expression of approximately 80 genes. Imprinted regions are marked with DNA methylation prior to repackaging of the paternal genome during spermiogenesis (Rousseaux et al., 2005). Imprinted genes subsequently regulate zygotic gene expression and embryonic development.

The sperm genome is highly methylated (89.4%) (Kobayashi et al., 2012). As an epigenetic mark, DNA methylation is vital in embryonic development where it regulates gene expression in a highly regulated spatial and temporal manner. The cytosine base contained with CpG dinucleotides designate methylatable sites within the genome, and are frequently found to be clustered into CpG islands (CGIs). CpG islands are found within promoter regions of approximately 70% of all annotated genes, and function to regulate transcription factor binding, and consequently, transcription of its associated gene(s) (Deaton and Bird, 2011). Genes associated with CGI-containing promoters include almost all house-keeping genes, a portion of tissue-specific genes and developmental regulator genes (as described in Deaton and Bird, 2011; Illingworth et al., 2008). Furthermore, CGIs that are remote from annotated transcription start sites (in intergenic or intragenic regions) have also been shown to demonstrate promoter function (Illingworth et al., 2010; Maunakea et al., 2010). Together, these findings emphasise the strong correlation between CGIs and transcription initiation.

The haploid genome of mice and humans contain a comparable number of CGIs (23,021 and 25,495, respectively) (Illingworth et al., 2010). The location of these CGIs are also highly conserved between mice and humans, with approximately 50% located in transcription start sites, and 25% in both intergenic and intragenic regions (Figure 7). The positions of many of these CGIs are conserved between mice and humans, which implies functional importance.

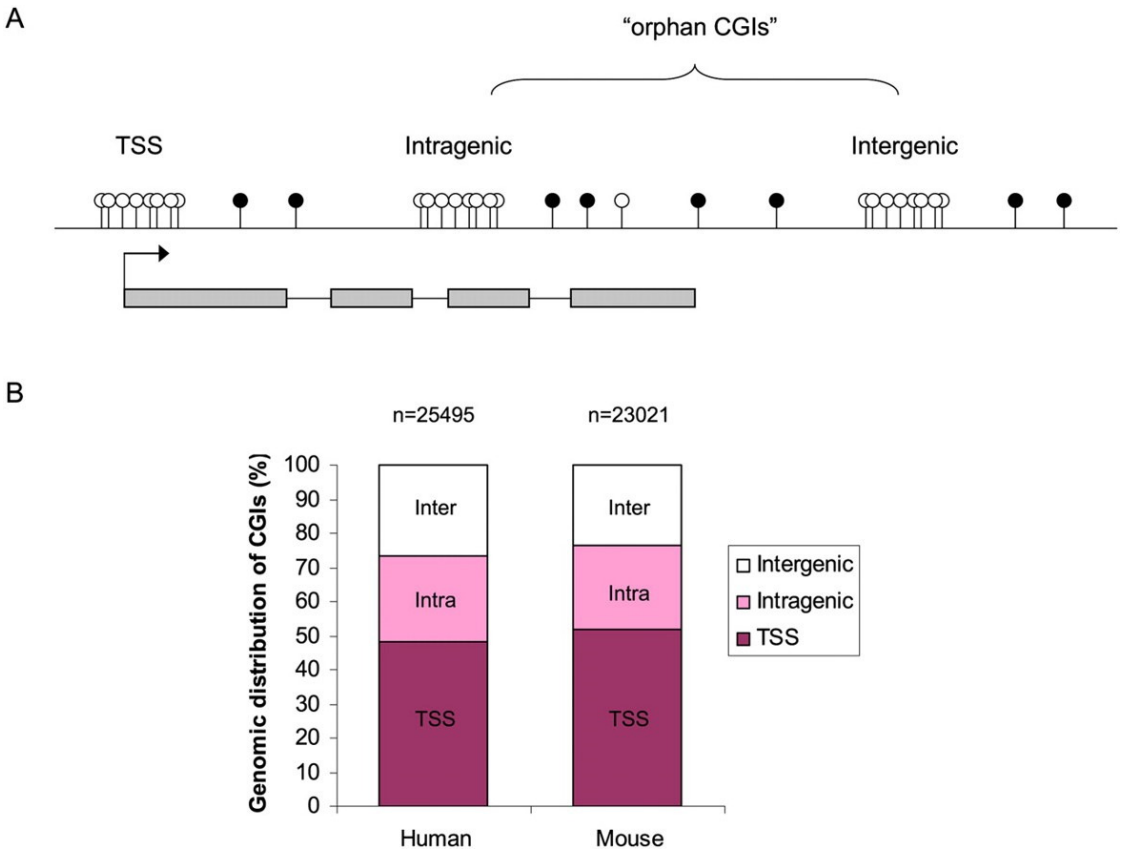


Figure 7: CpG island (CGI) distribution in the mouse and human genome. (A) CGIs are generally associated with transcription start sites (TSS), intragenic regions (gene bodies) and intergenic regions. (B) CGIs are commonly located in TTS (~50%), and ~25% in both intragenic and intergenic regions (Deaton and Bird, 2011; Illingworth et al., 2010).

Epigenetic signatures are established during the initial stages of gametogenesis in the developing embryo. Postnatally, from puberty into adulthood, spermatocytes undergo continual differentiation to form mature spermatozoa. During meiosis I and II, epigenetic marks are acquired, maintained and/or lost (Figure 8). These signatures include parent-of-origin specific imprints, methylation marks, histone modifications, and ncRNAs (Boissonnas et al., 2013).

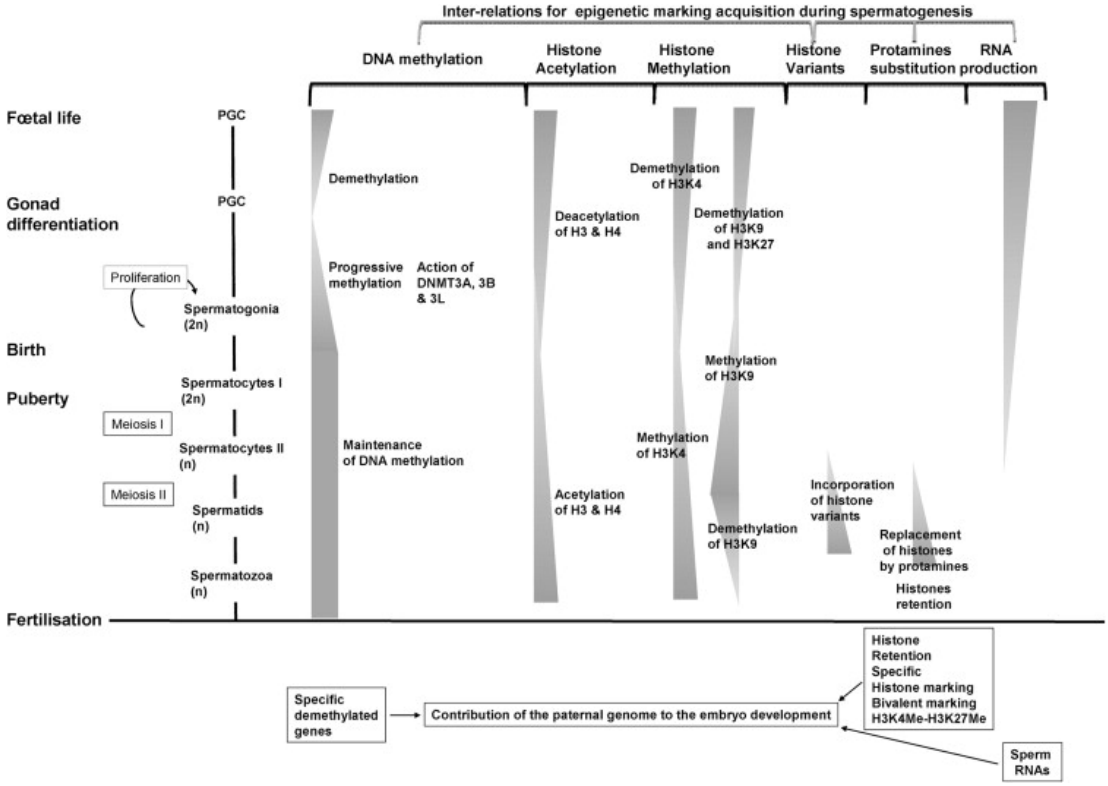


Figure 8: Epigenetic regulation during spermatogenesis. Epigenetic reprogramming during spermatogenesis dynamically involves DNA methylation, histone modifications, DNA packaging proteins (histones and protamines) and RNAs (Boissonnas et al., 2013).

1.3.3.2. Sperm haploid genome packaging

Nucleosomal histone complexes are capable of compacting genomic DNA by 10^5-fold by organising the chromatin into a highly folded and looped architecture in association with the nuclear scaffold (Razin et al., 2007). However, the smaller sperm nucleus requires a higher degree of compaction in order to package the haploid paternal genome. Sperm chromatin undergoes a tenfold (10^6-fold) compaction during the final stages of post-meiotic spermatogenesis (during spermiogenesis). This is achieved by replacing the vast majority of histones with protamines (Braun, 2001) through a process that is thought to be mediated by histone hyperacetylation (which dramatically reduces histone affinity to DNA) (McLay and Clarke, 2003). During this process, nucleosomes containing the octamer histone complex are

replaced initially with transition proteins and then by protamines (comprising protamine 1 and protamine 2). The stability of protamine-bound DNA is re-enforced by the establishment of inter- and intra-protamine disulphide bonds as the sperm matures during its passage along the epididymis (as reviewed in Miller et al., 2010). In this highly condensed state, the protamine-bound paternal genome is transcriptionally and translationally inert (as described in Miller et al., 2010) Following fertilisation, protamines are rapidly replaced by maternal histones derived from the ooplasm, forming the paternal pronucleus.

It is important to note however, that not all histones are replaced by protamines during the packaging of the paternal genome. Core histones are evident in mature spermatozoa, with humans and mice retaining 10-15% and 1%, respectively (Brykczynska et al., 2010; Gatewood et al., 1990; Hammoud et al., 2009). In particular, three sperm-specific core histone variants have been identified in elongating mouse spermatids and spermatozoa. These include H2AL1, H2AL2, and H2BL1 (Govin et al., 2007) Furthermore, it has been shown that histone-bound DNA in mature spermatozoa is retained in a peripheral location in mouse sperm nuclei (Pittoggi et al., 1999), and to the annular region in human sperm nuclei (Li et al., 2008). Evidence also indicates sequence composition differences between histone- and protamine-bound regions in human sperm nuclei, suggesting preference for packaging the paternal genome into different domains. Importantly, more open chromatin conformations (those which are nucleosome-bound) appear to favour sites for developmentally regulated genes (as described in Miller et al., 2010). This includes imprinted genes, HOX genes and developmental transcription and signalling factors (Miller et al., 2010). These loci have also been found to be associated with H3K4me3 and H3K4me2, which mark relaxed chromatin domains. Furthermore, developmental gene promoters in human sperm are hypomethylated. This is in contrast to the majority of sperm DNA, which is generally hypermethylated (Miller et al., 2010). Promoter sequences associated with, or in close

proximity to, CTCF binding sites also appear to be preferentially packaged in nucleosomal regions (Miller et al., 2010). Together, the association of nucleosomal regions with developmentally important genes and epigenetic marks that are generally associated with active transcription, suggests that regions of the paternal genome are primed for early activation following fertilisation. In support of this, sperm derived histones and associated modifications have been shown to contribute to zygotic chromatin formation during early embryonic development (Hammoud et al., 2009; van der Heijden et al., 2008), which are generally associated with key gene regulatory sequences, including CTCF binding sites (Saida et al., 2011).

During transit through the epididymis, spermatozoa mature. During this period, sperm are bathed in a gradient of RNases, glycosylases and proteases (as reviewed in Krawetz, 2005). This quality-control process serves to ensure the removal of any cellular component, including RNAs. However, the presence of RNA in maturing murine sperm was discovered in the 1970's (Betlach and Erickson, 1973), which, despite much scepticism, has been confirmed in plants (Engel et al., 2003) and other mammalian species (Paul and Duerksen, 1975), including humans (Kumar et al., 1993; Miller et al., 1999; Ostermeier et al., 2002). During the last transcriptional burst of spermatogenesis (during spermiogenesis, prior to transcriptional quiescence), several mRNAs are produced and subsequently stored in ribonucleoprotein particles (mRNPs). This effectively ensures the preservation of sperm RNA, protecting them from degradation (as reviewed in Krawetz, 2005). RNAs present in sperm represent a diversity of processes (Figure 9), and a comparison of transcript populations between ten individuals (Ostermeier et al., 2002) has revealed that all 2,780 transcripts (except four) were present in all ten populations, demonstrating that the preservation of RNAs is not a stochastic process. Several studies have subsequently demonstrated that the sperm contains, and delivers to the embryo, a unique milieu of non-coding RNAs that are

functionally important for embryonic development (Albertini, 2012; Carrell, 2012; Girard et al., 2006; Hamatani, 2012; Hammoud et al., 2011; Johnson et al., 2011; Krawetz et al., 2011; Miller, 2007; Miller et al., 2010; Miller and Ostermeier, 2006; Montjean et al., 2012; Ohnishi et al., 2010).

Overall, the spermatozoal epigenome contributes unique properties to the zygote, without which, developmental progression could be compromised.

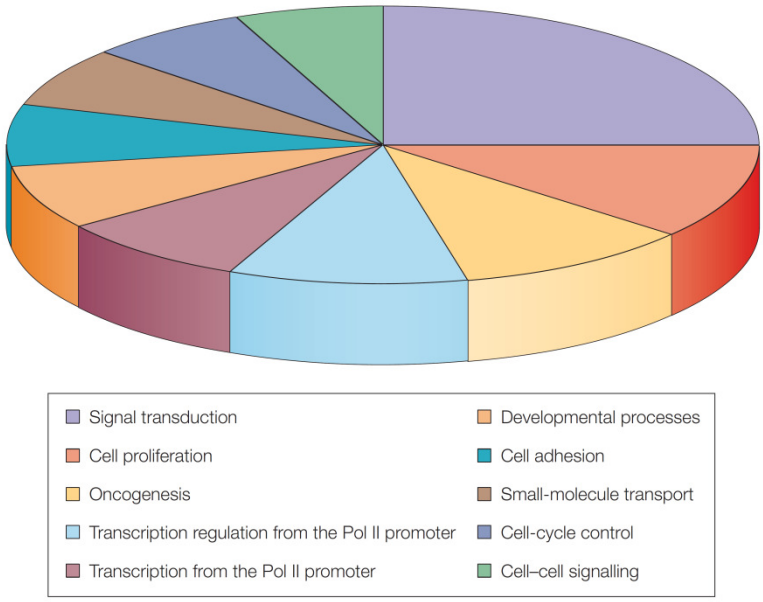


Figure 9: An ontology of biological processes that are represented by sperm RNAs. The pie chart shows the top-ten processes in which sperm RNAs participate. Sperm RNAs that are required for growth, signal transduction, cell proliferation, oncogenesis and transcriptional regulation from a polymerase II (Pol II) promoter are highly represented (Krawetz, 2005).

1.4. Epigenetic reprogramming

Epigenetic marks, systems and heritable "memories" that are laid down to regulate gene expression in a highly organised manner, need to be reset in order to restore a pluripotent state for the propagation of subsequent generations. This resetting of parental epigenetic signatures not only provides a blank canvas for developmental pathways to be re-established, but also allows the correction of epigenetic errors. It is thus vitally important that epigenetic information is correctly established in the parental gametes and maintained/orchestrated throughout development in a highly specified manner (Li, 2002).

Upon fertilisation of an egg, the early embryo has the ability to differentiate into all the tissues required for embryonic development. At the point of fertilisation, the parental genomes are in different stages of the cell cycle and possess very different epigenetic marks and chromatin organisation. A process known as epigenetic reprogramming removes established epigenetic marks present in the sperm and the oocyte genomes without altering the DNA sequence itself, returning the early zygote to an unprogrammed, pluripotent state. Subsequently, this process resets these marks and re-establishes specific gene expression programmes essential for cellular differentiation and embryonic development. This phenomenon involves the reprogramming of both histone modifications and DNA methylation (Figure 10, Figure 11, and Figure 12).

In mammals, reprogramming DNA methylation is a bimodal event (Figure 11), occurring during embryo preimplantation and gametogenesis (Reik et al., 2001). Upon fertilisation, the protamines present in the sperm genome are replaced with the histones H3 and H4, while the maternal genome completes meiosis. Following the deposition of histones, the paternal genome undergoes a genome wide loss of DNA methylation (Santos et al., 2002). This

process occurs before the initiation of DNA replication and importantly, excludes paternally methylated imprinted genes (Olek and Walter, 1997).

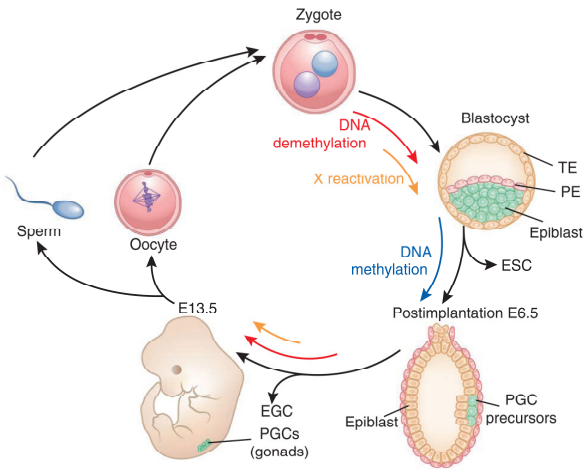


Figure 10: Epigenetic programming and reprogramming during the mouse life cycle. Epigenetic modifications are important for 'programming' lineage determination and cellular identity during development. Global 'reprogramming' of the epigenetic landscape instead marks the conversion of differentiated cells to totipotent or pluripotent states, upon fusion of the gametes (sperm and oocyte) in the zygote and in the PGCs after their specification from the somatic epiblast of the postimplantation embryo. It is notable that two populations of pluripotent cells can be established *ex vivo* within the time window in which extensive epigenetic reprogramming takes place. These cells are ESCs and embryonic germ cells (EGCs) that are derived from the inner cell mass of the blastocyst and from the PGCs at E8.5–E13.5, respectively. Major remodeling events (for example, DNA demethylation and X-chromosome reactivation) are highlighted in the figure by colored arrows. TE, trophoectoderm; PE primitive endoderm (Cantone and Fisher, 2013).

1.4.1. Reprogramming of the zygote

Further changes in global DNA methylation and histone modifications occur once the fertilised egg starts to divide. From the one cell stage of the mouse blastocyst, extensive DNA methylation erasure occurs in the zygote through the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), as well as through a passive mechanism where

successive rounds of cellular division and DNA replication result in unequally methylated sister chromatids (Wossidlo et al., 2011). At the morula stage of embryogenesis, lineage allocation occurs and the trophectoderm and inner cell mass (ICM) lineages are defined. Reprogramming of CpG methylation in the pre-implantation embryo results in a globally demethylated genome in the inner cell mass (ICM), and is complete by approximately E3.5 (Hackett and Surani, 2013). At this stage, pluripotent cells are established, which give rise to embryonic stem cells. Following 5mC erasure, *de novo* remethylation begins at implantation. Re-establishment of 5mC is directed by Dnmt3a and Dnmt3b in the ICM, and is mostly complete by approximately E6.5 (Smith et al., 2012). At this point, global differences in DNA methylation between extraembryonic (placental) and embryonic lineages become apparent. Recent evidence suggests that progressive acquisition of developmental DNA methylation occurs at key promoters, and it has been proposed that this acquisition of promoter CpG methylation occurs at an early developmental stage to define initial lineage-restriction (Hackett and Surani, 2013). It is important to note however, that despite the global wave of demethylation that occurs in the early zygote, imprinted regions escape demethylation and retain their methylation status in a parent-of-origin specific manner and are maintained throughout embryonic development.

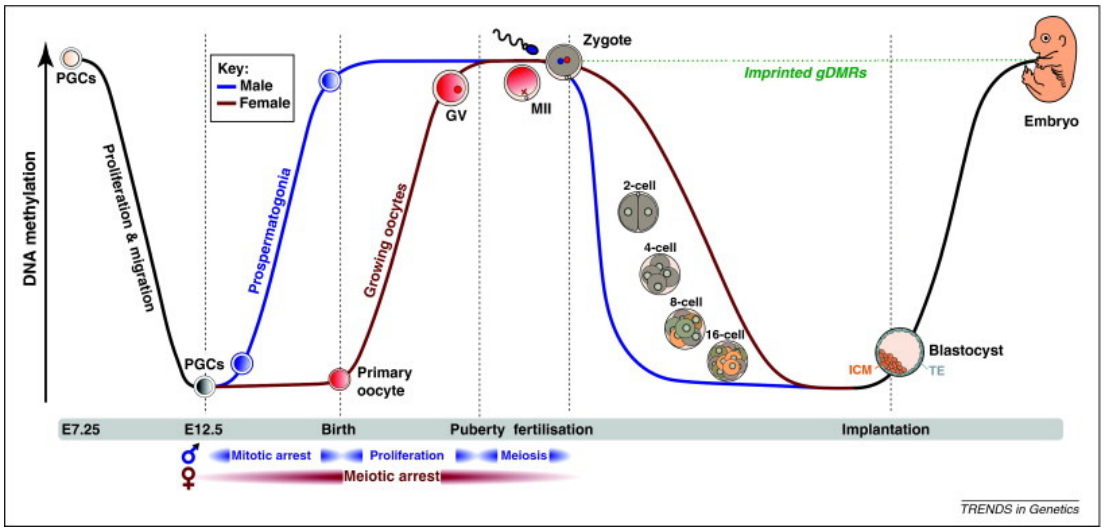


Figure 11: DNA methylation changes during developmental epigenetic reprogramming. (Smallwood and Kelsey, 2012)

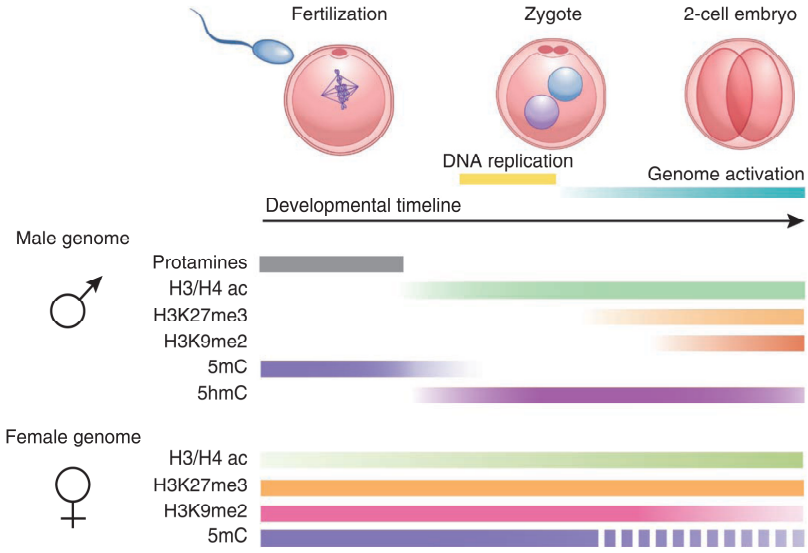


Figure 12: Epigenetic changes during in vivo reprogramming. Schematic of global DNA and histone modifications that lead to transcriptional activation of the embryonic genome between the late zygote (paternal genome only) and the 2-cell stage. Gamete genomes undergo different epigenetic programs after fertilization with the paternal genome being mostly subject to epigenetic remodelling at the zygote stage and the maternal genome gradually losing repressive modifications during the subsequent cleavage divisions (Cantone and Fisher, 2013).

1.4.2. Reprogramming of the primordial germ cells *in utero*

The second wave of epigenetic reprogramming that occurs during embryonic development involves the primordial germ cells (PGCs). This second reprogramming event is important, as it erases parent-of-origin dependent genomic imprints, and confers plasticity to the gamete genomes (Hackett et al., 2012). At approximately E7.25, mouse PGCs derived from epiblast cells (Ginsburg et al., 1990) migrate to, and reach, the genital ridge by embryonic day 11.5 (E11.5). Prior to the onset of sexual differentiation, both XX and XY PGCs that have migrated to the genital ridge by E11.5, and show strong expression of *Dnmt1*, which is primarily located in the nucleus (La Salle et al., 2004). These PGCs colonising the embryonic gonad contain epiblast-derived epigenetic marks which are subsequently erased during the second reprogramming event between E10.5 and E12.5 (Figure 13). Epiblast-derived DNA methylation marks are completely removed through active demethylation by E12.5. Unlike in embryonic somatic tissue, genome-wide erasure of DNA methylation in PGCs includes imprinted regions, but excludes some repetitive elements, particularly the LTR of Intracisternal A Particle (IAP) (Hajkova et al., 2002). Reprogramming of primordial germ cells is critical as it renders the epigenetic state of germ cells of both sexes equivalent. *Dnmt1* is once again expressed in the E12.5 genital ridge germ cells (Hajkova et al., 2002), and suggests an important role of this methyltransferase in maintaining the methylated (silent) state of repetitive elements in mitotic PGCs. Sexual differentiation of mouse PGCs occurs during E12.5 and E18.5, where *Dnmt1* continues to be expressed in both dividing male and female germ cells (Figure 14). During sexual differentiation of the foetal testis, *Dnmt1* is highly expressed during E13.5 and E18.5 – a time during which global DNA methylation is re-established. This re-methylation event occurs earlier in the male germline, at the prospermatogonia stage (E15 – E16) (Reik et al.). This re-methylation event results in a highly

methylated (sperm) or a partially methylated (oocyte) genome (Smallwood and Kelsey, 2012). This second 5mC reprogramming event ensures that unique epigenome signatures are established in PGCs, which enables their differentiation into mature gametes. Prior to birth, male PGCs enter mitotic arrest and genomic methylation begins to be acquired in prospermatogonia (La Salle et al., 2004).

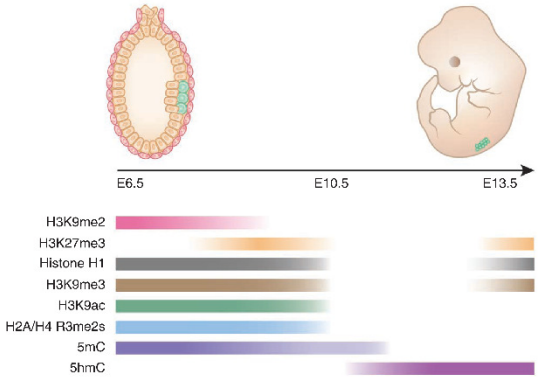


Figure 13: Epigenetic changes during in epigenetic reprogramming. Global epigenetic changes during germline development in the from PGC specification (E6.5) to mitotic/meiotic arrest at E13.5. Two major epigenetic phases can be distinguished during PGC migration toward the genital ridges (E7.5-E10.5) and upon their arrival into the gonads (E10.5-E12.5) (Cantone and Fisher).

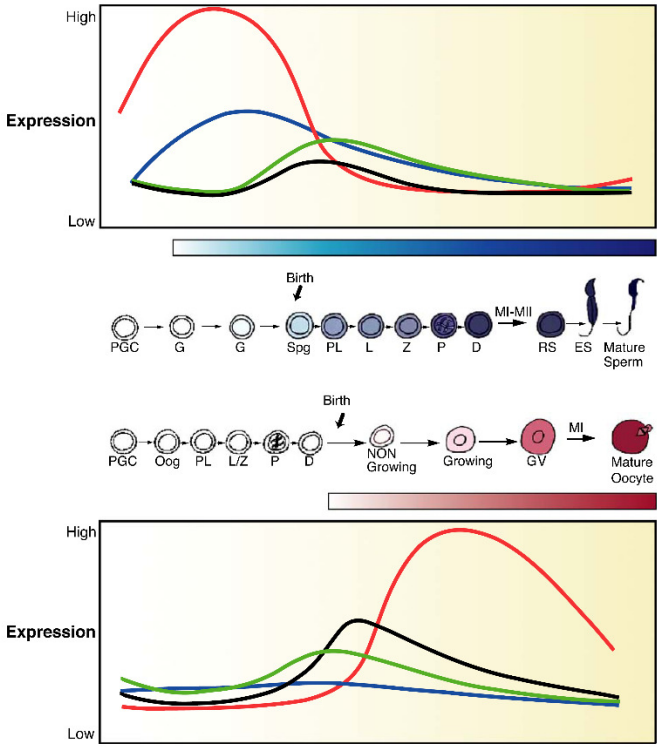
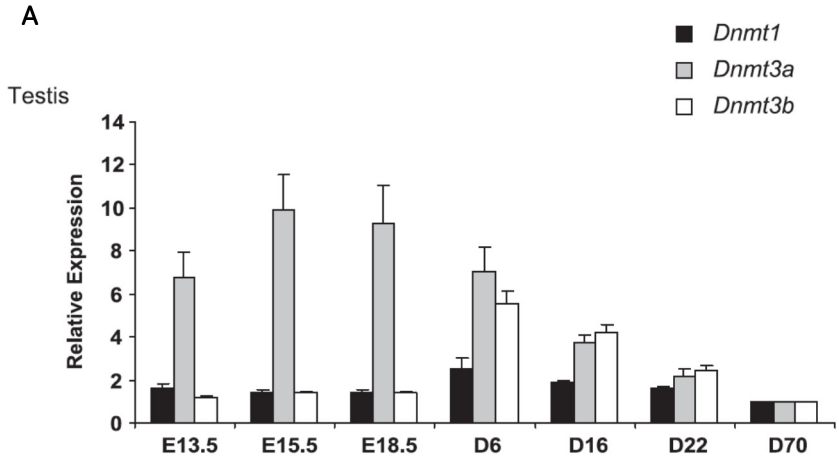


Figure 14: Expression levels of *Dnmts* during gametogenesis Schematic representation of DNA methyltransferase dynamics during mouse gametogenesis. Progression of the establishment of methylation marks at imprinted and non-imprinted loci and relative levels of Dnmt1 (black), Dnmt3a (blue), Dnmt3b (green), and Dnmt3l (red) are presented as functions of spermatogenesis and oogenesis. Intensity of the shading in the boxes reflects the methylation status of the paternal (blue) and maternal (red) genomes. Dnmt3l levels are not to scale. Representative cell types are indicated as follows: PGC, primordial germ cell; G, gonocyte; Spg, spermatogonia; Oogoogonia; PL, preleptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene; GV, germinal vesicle; RS, round spermatid; ES, elongating spermatid. MI– MII, meiosis I – II (La Salle et al., 2004).

1.4.3. Reprogramming in the postnatal gametes: spermatogenesis

At birth (0 dpp), the mouse prospermatogonia in the neonatal testes show no evidence of Dnmt1. The onset of postnatal mitosis of the germ cells occurs between 3 and 4 dpp, where spermatogonial stem cells resume mitosis (Takayama et al., 2005). At this point, the germ cells have relocated to the basal compartment of the seminiferous epithelium.

Subsequently, *Dnmt1* expression is highly elevated in the dividing spermatogonia at 6 dpp (La Salle et al., 2004). Genomic methylation of the male gametes is completed after birth, before the end of pachytene (La Salle et al., 2004). In contrast, in female germ cells, methylation patterns are acquired postnatally, following the pachytene phase of meiosis is complete, during the oocyte growth phase (as reviewed in La Salle et al., 2004). During spermatogenesis, when spermatids mature to spermatozoa, a wave of *de novo* methylation by Dnmts establishes paternal specific imprints (Marques et al., 2011). *Dnmt3a* expression in the testes is prominent before and shortly after birth, and gradually reaches basal levels by postnatal day 70 (Figure 15). In contrast, *Dnmt3b* levels are low before birth, and increase at postnatal day 6 and 16 (Figure 15). *Dnmt3L* is highly expressed in the prenatal testis, and shows a marked decrease following birth (Figure 15). Overall, this evidence suggests that Dnmt3b and Dnmt3L play a role in the postnatal testis in the maintenance of methylation marks in the rapidly dividing spermatogonia.



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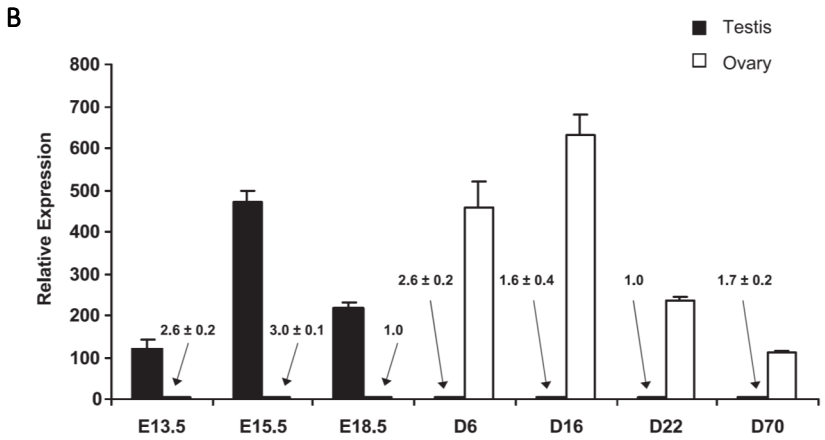


Figure 15: Expression of *Dnmts* in the mouse gonads. Expression dynamics of DNA methyltransferases in the developing testis and ovary. (A) Relative quantification of Dnmt1 (black bar), Dnmt3a (gray bar), and Dnmt3b (white bar) in the testis. (B) Dnmt3L expression in the testis and ovary. E=embryonic day, D=postnatal day (La Salle et al., 2004).

The process of spermatogenesis occurs following sexual maturity, and produces haploid spermatozoa from diploid spermatogonial stem cells. In this process, the spermatogonial stem cells transition through the seminiferous epithelium to become haploid spermatozoa. The seminiferous epithelium is comprised of concentric layers of germ cells which are surrounded by Sertoli cells, which together, form the seminiferous tubules (Cooke and Saunders, 2002). The process of spermatogenesis in the mouse is divided into XII stages, which can be grouped into early (Stages I-V), middle (VI-VIII), and late (IX-XII) stages.

At the onset of spermatogenesis, the diploid germ cells (spermatogonia) undergo several rounds of mitosis within the basement membrane. B-type spermatogonia subsequently undergo mitotic divisions, and give rise to two preleptotene spermatocytes. *DNMT1* is expressed in preleptotene, leptotene, and zygotene spermatocytes before being down-regulated at pachytene (Jue et al., 1995; Marques et al., 2011). Maintenance methylation by Dnmt1 continues throughout spermatogenesis post sexual maturity (Boissonnas et al., 2013).

Meiotic cell division occurs in, and defines, Stage XII. During this stage, the primary spermatocyte undergoes meiosis I (2n), generating two secondary spermatocytes (n). This is followed by meiosis II, which forms two round haploid spermatids per secondary spermatocyte. The duration of spermatogenesis varies greatly among mammals. In the human, spermatogenesis is complete within 72 days, while the total duration is approximately 39 days in the mouse (Franca et al., 2005).

Following the mitotic and meiotic phases of spermatogenesis, the round spermatids become elongated, highly condensed, and mature. This process is known as spermiogenesis (Franca et al., 2005). Re-methylation, and the establishment of paternal germline-specific methylation signatures, occurs during sperm maturation in the epididymis (Ariel et al., 1994).

Between the onset of de novo methylation and the production of mature sperm, there are multiple rounds of cell division, such that initially determined methylation patterns may be modified through maintenance, and there is greater opportunity for methylation errors to accumulate (Kelsey and Feil, 2013)

1.5. Effect of environmental exposures on epigenetic signatures

1.5.1. Effect on DNA methylation of the developing embryo

Several studies have shown that nutritional influences in early life can induce permanent alterations in the epigenotype and determine adult phenotypes and disease susceptibility. Several groups have investigated whether maternal diets can alter the coat colour of viable

yellow agouti (*A^{vy}*) mice depending on the epigenetic state of the retro-transposon. It is well established that epigenetic processes are highly dependent on the dietary availability of key nutrients, for example methyl donors and cofactors. When A^{vy} pregnant dams were fed a diet supplemented with methyl donors and cofactors (e.g. choline, folic acid, vitamin B12) they tended to have more offspring that were pseudo-agouti and lean rather than being yellow and obese, as a result of increasing the level of methylation at the A^{vy} allele. This demonstrates that the epigenetic state of a transposable element downstream of the *Agouti* locus can be influenced by maternal diet, as described here for the A^{vy} allele and more recently in mice that carry a transposable insertion at the *Axin* gene (Ozanne and Constancia, 2007). In humans, Heijmans et al. (2008) reported hypomethylation of the imprinted *IGF2* gene in genomic DNA isolated from whole blood from individuals who were exposed to famine *in utero* during the Dutch Hunger Winter compared with unexposed same-sex siblings. The same group also found that *IGF2* was hypomethylated in individuals whose mothers were periconceptually exposed to famine, whereas interleukin-10, leptin, ATP-binding cassette A1, guanine nucleotide-binding protein and maternally expressed 3 (*meg3*) were hypermethylated (Tobi et al., 2009). Furthermore, the collective data from mouse models of nutritional constraint and uteroplacental insufficiency would suggest that the gestational milieu profoundly influences the postnatal phenotype to render an increased susceptibility to childhood obesity via *in utero* alterations in foetal histone H3 covalent modification (Aagaard-Tillery et al., 2008; Delage and Dashwood, 2008).

In addition to nutritional effects, toxin exposure (Anway et al., 2008), intrauterine environment (Lim and Ferguson-Smith, 2010), trace elements (Vahter, 2007), and hormone treatment (Pathak et al., 2011) have been shown to affect epigenetic signatures and offspring outcome.

Together these findings demonstrate that external stimuli can affect epigenetic mechanisms *in utero* to such an extent that foetal programming is perturbed and manifests in later increased risk for disease phenotypes.

1.5.1.1. Foetal Alcohol Spectrum Disorders

Prolonged levels of maternal alcohol exposure during pregnancy have been implicated in a range of unfavourable foetal outcomes, collectively known as Foetal Alcohol Spectrum Disorders or FASD (Burd et al., 2003), with the most severe of these outcomes being Foetal Alcohol Syndrome (FAS). FAS can manifest with variable phenotypes resulting in diverse combinations of craniofacial, growth and neurobehavioral abnormalities associated with a multitude of symptoms and psycho-social problems. These include attention deficit-hyperactivity disorder (ADHD), learning disabilities, behavioural disorders (Burd et al., 2003), facial dysmorphia, reduced body growth and central nervous system abnormalities, of which mental retardation is the most common (O'Leary, 2004). The quintessential characteristics of FAS which all diagnoses are based upon are prenatal and postnatal growth retardation, mental disability and the distinct craniofacial abnormalities, which include a thin upper lip, smooth philtrum and a flat nasal bridge.

Drinking during pregnancy remains a problem worldwide. Roughly 15% of American women of childbearing age are regarded as moderate to heavy drinkers, and nearly 2% to 3% are at risk of dangerously exposing their unborn child to the adverse effects of alcohol (Cordero et al., 2004). The Centre for Disease Control has reported prevalence rates of 0.2 to 1.5 cases per 1000 live births in the United States (Cordero et al., 2004). While an average FAS rate of 0.97 per 1000 children has been reported globally, a staggering rate of 65.2 - 89.2 per 1000 children – the highest world-wide – has been reported in the Western Cape, South Africa

(May et al., 2000; May et al., 2007; Viljoen et al., 2005). This statistic emphasizes the importance, if not the necessity of foetal alcohol related research, especially in South Africa.

1.5.1.2. The biological effects of alcohol

The volume and pattern of alcohol consumption lead to three important sequelae that directly impact disease and injury. These are (1) direct cytotoxic effects of alcohol on organs and tissues; (2) intoxication; and (3) dependence (Rehm et al., 2003).

Alcohol is a well-known teratogen which causes developmental abnormalities in the foetus (Boehm et al., 1997; Camarillo and Miranda, 2008; Ogawa et al., 2005; Randall, 1987; Randall et al., 1994). These teratogenic effects have been studied in both humans and in animal models. As described previously, researchers have shown that foetal exposure to excessive levels of alcohol can result in a wide variety of neurobehavioural deficits, primary of which include central nervous system dysfunction, growth deficiencies and distinct facial anomalies, with varying degrees of severity.

Many mechanisms have been proposed in an attempt to explain the teratogenic effects of alcohol on the developing foetus. In addition to the fact that alcohol itself has the ability to act as a direct cytotoxic agent (De Vito et al., 2000), the metabolic products that arise from its subsequent breakdown in the body play a significant role in alcohol's deleterious effects (Fernandez-Checa, 2003; Poggi et al., 2003). This therefore highlights the fact that there is no single mechanism that accounts for the range and extent of phenotypes evident in children born with foetal alcohol effects as a result of being exposed to significantly high levels of alcohol. As the focus of this research is on paternal effects of excessive alcohol intake, the direct effects of this teratogen on the developing foetus will not be discussed

further, but the emphasis will be on the metabolic aspects of alcohol and its potential effect on male gametogenesis.

1.5.1.3. The effects of alcohol metabolism

Recent reviews (Halsted et al., 2002; Schalinske and Nieman, 2005) have highlighted that the metabolism of alcohol and the interplay of its metabolites on the folate, methionine and homocysteine pathways. The metabolism of these three compounds is intricately connected and is vital for the maintenance of optimal health (Wagner, 1995). It has been shown that folate deficiency is a common clinical sign of chronic alcohol abuse and has been implicated in the development of alcoholism-related complications, such as alcoholic liver disease (Eichner et al., 1971). Researchers have also found that disruption of this pathway has been associated with various disorders including, among others, birth defects and neurological abnormalities (Boyles et al., 2006).

To understand the biological importance and therefore pathologies associated with disrupted folate, methionine and homocysteine metabolism, a closer inspection of these processes needs to be considered. As a coenzyme, folate is required to supply methyl groups for S-adenosylmethionine (SAM)-dependent transmethylation reactions. In the folate-dependent pathway, the B_{12}-dependent enzyme methionine synthase (MS) is responsible for transferring the methyl group contained within the 5-methyl-tetrahyrofolate compound to homocysteine (Figure 16).

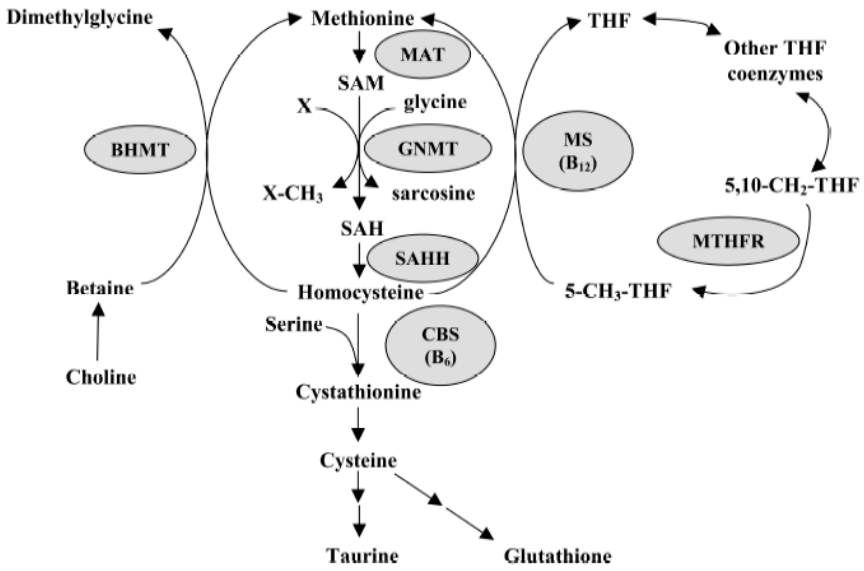


Figure 16: One-carbon metabolism. (Schalinske and Nieman, 2005)

As early as 1974, research on alcohol-fed rats described reduced MS activity and subsequent reduction of the by-products methionine and SAM (Barak et al., 1987; Finkelstein, 1974; Trimble et al., 1993). It has also been shown that ethanol appears to enhance the catabolism and loss of methyl groups, which in turn disrupts subsequent SAM-dependent transmethylation reactions (Schalinske and Nieman, 2005). Cancer related research has demonstrated that perturbations to these reactions are known to result in hypomethylation of hepatic DNA (Bhave et al., 1988; Wainfan et al., 1989).

Alcohol has also been shown to affect methionine, folate and choline – all of which play a role in the production of SAM. This effect is subsequently able to influence histone modifications, ncRNAs, and DNA methylation (Figure 17) (Resendiz et al., 2013).

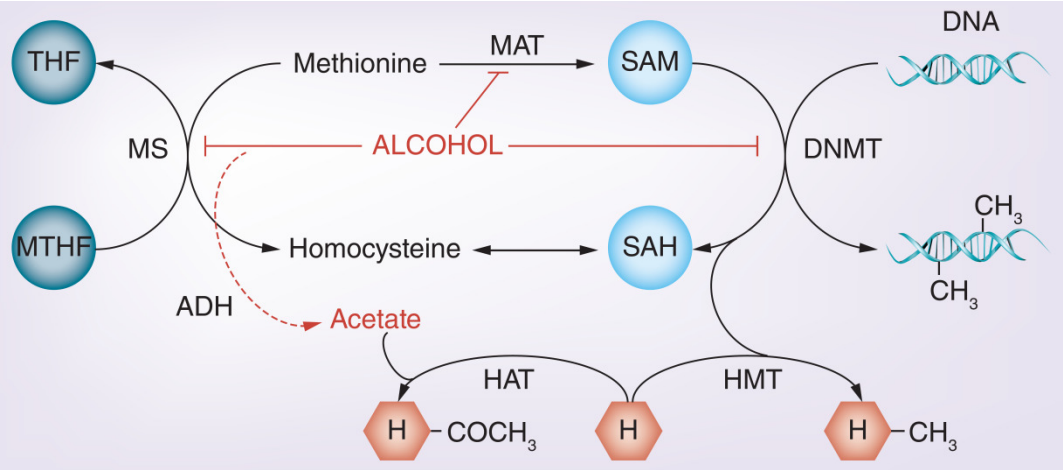


Figure 17: The biological effect of alcohol on epigenetic marks. DNA and histone methylation occurs mainly through the transfer of a methyl group to the substrate. SAM actively carries the methyl donor group. SAM is synthesized from methionine, which itself can be produced from the dietary intake of folate, choline and other methyl donors (only folate is shown here for simplicity). Alcohol can affect the methionine synthesis process by inhibiting metabolic enzymes, MAT and DNMTs, both through direct and indirect processes. This consequently induces a decrease in SAM production and hyperhomocysteinemia. In addition, acetate, a by-product of alcohol metabolism (indicated by a dashed arrow), is involved in the acetylation of Hs. DNMT: DNA methyl transferase; H: Histone; HAT: Histone acetyltransferase; HMT: Histone methyltransferase; MTHF: Methyltetrahydrofolate; SAH: *S*-adenosylhomocysteine; SAM: *S*-adenosylmethionine; THF: Tetrahydrofolate (Resendiz et al., 2013).

1.5.1.4. An epigenetic model of FAS

DNA methylation is a vital regulator of gene expression. At imprinted loci, it determines the binding of the insulator protein CTCF, which in turn regulates the expression of associated genes within the locus in a parent-of-origin-specific manner; while at promoter regions and enhancers, DNA methylation influences the binding of various transcription factors and other regulatory molecules.

Aberrations in DNA methylation of imprinted regions, and in particular ICRs, have been associated with developmental disorders, including AS, PWS and BWS, which are associated with abnormal growth phenotypes and neurological deficits. The cause of aberrant DNA

methylation at ICRs, such as that of *H19* in Silver Russell Syndrome, remains largely unknown. However, several environmental factors have been associated with decreased methylation in these regions. Heavy metals (Aina et al., 2004), pesticides (Anway and Skinner, 2006), cigarette smoke (Kaplan et al., 2003), dietary folate deficiencies (Friso and Choi, 2002) and alcohol have all been associated with DNA methylation disruption. Alcohol and dietary folate intake have been linked with cancer, where alcohol-fed rats displayed disruption of the folate, methyl group and homocysteine metabolism pathway, with subsequent reduction of the by-products essential for DNA methylation (Barak et al., 1987; Finkelstein, 1974; Trimble et al., 1993). Perturbations to these metabolic pathways result in hypomethylation of hepatic DNA, and are associated with hepatomas in the rat liver (Bhave et al., 1988; Wainfan et al., 1989). Low folate/high alcohol intake has been associated with sporadic colorectal cancer (Giovannucci et al., 1995; van Engeland et al., 2003), and upper alimentary and liver cancer (Poschl and Seitz, 2004).

Alcohol has also been shown to alter methylation involved in developmental pathways. Prolonged levels of maternal alcohol exposure during pregnancy have been implicated in a range of unfavourable foetal outcomes, such as FASD. FASD is characterised by hyperactivity and reduced cognitive function. Evidence shows that folate is associated with both of these FASD hallmarks (Schlotz et al., 2010; Veena et al., 2010). This has been further supported by findings whereby alcohol consumption during pregnancy creates oxidative stress to both the placenta and foetus, which can be mitigated by folic acid (Cano et al., 2001; Gundogan et al., 2010) Investigations using a mouse model into the effects of alcohol on the methylation signatures of imprinted genes have revealed that excessive exposure resulted in global hypomethylation of foetal DNA, providing a link between loss of DNA methylation and FAS (Garro et al., 1991). Several animal models have supported the association of prenatal alcohol exposure and perturbations to DNA methylation, both locally at specific genes

(Haycock and Ramsay, 2009; Stouder et al., 2011), gene promoters (van Engeland et al., 2003) and on a genome-wide scale (Choi et al., 1999; Kaminen-Ahola et al., 2010; Liu et al., 2009; Zhou et al., 2011b).

1.5.2. Effect on male germline DNA methylation

Epigenetic signatures are mitotically and meiotically inherited as they are faithfully replicated during cellular division. That is, DNA methylation signatures on a parental strand of duplicated DNA is used as a template to guide the establishment of DNA methylation on the daughter strand by Dnmt1 in order to maintain these signatures during cellular division.

Epigenetic aberrations, termed "epimutations", to the parental or template DNA can also be perpetuated in the same manner. The common Toadflax (*Linaria vulgaris*) typically displays a bilateral symmetry. However, an epigenetic mutation to the *Lcyc* promoter region, which renders it highly methylated and transcriptionally silent, changes the symmetry of the Toadflax flower to radial (Cubas et al., 1999). This epimutation was subsequently inherited transgenerationally, where F2 plants displayed radial symmetry. This transgenerational inheritance shows evidence of a transmissible epimutation that is propagated through the germline.

Germline transmission of epimutations has also been demonstrated in animal models. *Axin-fused* (*Axin^{Fu}*) is a dominant gain-of-function allele in mice that manifests as a kink in the tail, and is variably expressed. *Axin^{Fu}* contains an interstitial A particle (IAP) retrostransposon insertion, which contains a long terminal repeat (LTR) that is variably methylated. Hypomethylation of the IAP results in a kinked tail phenotype, while normal mice have a hypermethylated IAP, which is transmissible through parental lineages (Rakyan et al., 2003).

Exposure of the unborn foetus to an environmental agent that is able to induce epimutations, not only affects the somatic tissue of the F1 foetus, but also the developing germline of the foetus. The germline of the foetus will ultimately give rise to F1 gametes, and through reproduction, give rise to the F2 generation. Thus, the gametes of the exposed F1 individual are essentially the genetic contribution to the F2 generation (Figure 18), and have profound implications for transgenerational inheritance of epimutations. The viable yellow agouti mouse model has been typically used to study the influence of environmental exposures on foetal development. In the wild-type state, the *Agouti* gene produces a brown coat colour in mice (a combination of black eumelanin and yellow eumelanin) (Dolinoy, 2008). An insertion of an IAP upstream, of the *Agouti* gene results in the epigenetically regulated metastable allele (epiallele), A^{vy} (Waterland and Jirtle, 2003). In the hypomethylated IAP state, *Agouti* is ectopically expressed, and produces a yellow coat colour. In the hypermethylated state, the Agouti gene is expressed normally and produces a brown coat colour (Duhl et al., 1994). Methyl donor supplementation of pregnant mice alters DNA methylation at the A^{vy} locus (IAP), which shifts the coat colour of offspring from yellow to the brown (pseudoagouti) phenotype (Wolff et al., 1998). Moreover, these effects were transgenerationally inherited through the germline to the F2 generation despite the advent of epigenetic reprogramming (Cropley et al., 2006).

Evidence of germline epigenetic insult and transgenerational inheritance is also evident in humans. In particular, individuals who were prenatally exposure to famine during the Dutch Hunger Winter of 1944/1945 were shown to have less DNA methylation at the *IGF2* imprinted gene when compared to that of same-sex unexposed siblings (Heijmans et al., 2008). To investigate the effects of food supply and smoking on the growth of subsequent generations, Pembrey et al (2006) studied the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort and the Överkalix cohort from Sweden (who historically suffered

food supply shortages from 1890-1920). They demonstrated that early paternal smoking was associated with greater body mass index (BMI) in 9 year old sons from the ALSPAC group, but not daughters. The Överkalix cohort revealed that paternal grandparental food supply was only linked to mortality rate of grandsons, while paternal grandmother's food supply was associated with mortality rates in granddaughters. Further to this, they demonstrated that nutrient deficiency of parents and grandparents during their slow growth period was associated with cardiovascular and diabetes mortality of subsequent generations (Kaati et al., 2002). Together, these human studies provide evidence for paternal germline transmission of environmentally-induced epimutations.

The ability of environmental exposures to induce epimutations to the male germline has been further demonstrated in animal models. However, these studies are limited. Vinclozolin has been shown to alter the sperm epigenome (Guerrero-Bosagna et al., 2010) and DNA methylation at imprinted genes (Stouder and Paoloni-Giacobino, 2010). Long-term exposure to butyl paraben induce DNA hypermethylation in the germ cells of the testes of male rats (Park et al., 2012). In addition to vinclozolin, alcohol exposure has been shown to affect DNA methylation at the *H19* ICR region of male mice exposed *in utero* (Stouder et al., 2011).

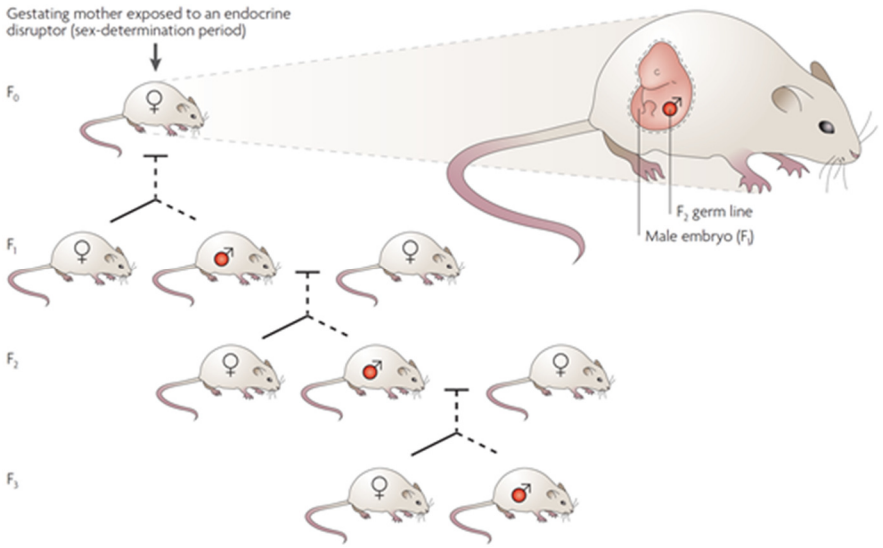


Figure 18: Germline transmission of epimutations. (Jirtle and Skinner, 2007)

Evidence of postnatal effects of the environment on sperm DNA methylation is even more scarce. Doshi et al (2012) observed that neonatal rats exposed to bisphenol A down regulates DNA methyltransferase activity and related transcription factors in resorbed embryos. These authors suggest that bisphenol A may have altered the sperm epigenome and consequently affected embryonic development and lead to embryo resorption. Ouko et al (2009) noted subtle, yet significant demethylation of the *IG*-DMR in the sperm of males who consumed alcohol regularly.

Evidence for the inheritance of an epimutation that is causal of a disease in human offspring has been only fairly recently discovered, and extensively debated. In 2004, Suter et al (2004) reported two individuals with soma-wide, allele-specific and mosaic hypermethylation of the DNA mismatch repair gene *MLH1*. These patients had multiple primary tumours that showed mismatch repair deficiency, meeting the clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC). However, there was no evidence to suggest a genetic mutation of

any mismatch repair gene. Epimutations in the *MLH1* gene have been associated with HNPCC (Hitchins and Ward, 2009). Suter et al demonstrated an epimutation in MLH1, which was also present in spermatozoa of one of the individuals, indicating a germline defect and the potential for transmission to offspring. However, this was partially retracted in 2007 (Hitchins and Ward, 2007), as these authors failed to confidently prove *bona fide* transgenerational epigenetic inheritance. Despite the debate over *bona fide* transgenerational epigenetic inheritance at *MLH1*, these authors did show that the MLH1 epimutation was at least transmissible to the F1 generation, which was later reaffirmed (Hitchins et al., 2007). More recently, transgenerational inheritance of the *MLH1* epimutation has gained support (Crepin et al., 2012). Similar findings of a heritable epimutation were observed in the *MSH2* gene which is also associated with HNPCC (Chan et al., 2006).

Part of the debate over the transgenerational inheritance of the MLH1 epimutation was around the fact that during gametogenesis, epigenetic marks (including DNA methylation) are erased and re-established. Albeit it true for the most part, this doctrine has been contradicted through several studies, which show that epigenetic marks are in some instances, able to escape this reprogramming. Some of these studies have been discussed previously, and is highlighted by Stouder et al (2011) who demonstrated that ethanol exposure in utero can alter sperm methylation, which can be inherited, and is suggestive of the fact that *H19* escapes reprogramming. More recently, Govorko et al (2012) demonstrated the persistence of epigenetic (DNA methylation) changes at the *POMC* promoter across the male germline. Guerrero-Bosagna and Skinner (2014) also put forward a compelling argument as to how environmentally-induced epimutations can be propagated and inherited in subsequent offspring. They posit that during the initiation of development of the germline, a major DNA methylation erasure occurs followed by the reestablishment of

DNA methylation patterns. This period in germ cell development and epigenetic programming represents a window of sensitivity to environmental factors, and when an altered epigenetic programming is induced, it can be perpetuated across generations. Paternal transmission of aberrant epigenetic states has been supported by Zeybel et al (2012), who demonstrated transmission of a heritable phenotype (wound healing) via epigenetic mechanisms (DNA methylation and histone acetylation) through the sperm, which resulted in reduced generation of liver myofibroblasts, increased hepatic expression of antifibrogenic PPAR-ɣ and decreased expression of profibrogenic TGF-ß1.

Overall, epigenetic reprogramming during gametogenesis provides a vulnerable window for environmental factors to exert their effect. In particular, alcohol is able to decrease the methylation available for transmethylation reactions vital for establishing DNA methylation signatures at imprinted regions and promoter regions. Paternal alcohol exposure has been associated with reduced *Dnmt* activity (Bielawski et al., 2002) and DNA methylation within the male germline, which is potentially transmissible. Furthermore, evidence exists that shows that environmental exposures are able to induce epimutations in the male germline, and that these epimutations are transmissible. Moreover, it has been demonstrated that paternal environmental exposures can impact on the development and health of subsequent offspring. However, there appears to be a lack of evidence for a mechanism of this transmission. That is, to date there has not been a study that shows definitive evidence of an environmentally-mediated effect on the male gamete epigenome, which is transmissible to subsequent offspring, which is affected to such an extent, that the epigenetic changes can elicit a change in gene expression, and manifest phenotypically.

Our previous study demonstrated that the effect of alcohol on DNA methylation at imprinted regions in the sperm of chronically exposed male mice, is at best, nuanced (Knezovich and Ramsay, 2012), and may be supported by others (Stouder et al., 2011). Despite this failure to observe significant changes in sperm DNA methylation, our previous study did observe significant decreases in DNA methylation at these same imprinted regions in the offspring sired by ethanol exposed males, which was associated with a window of postnatal growth restriction. The apparent lack of an initial epigenetic insult in the sperm, but evidence of an epigenetic alteration at the same loci in sired offspring is perplexing. This may suggest that epigenetic states other than DNA methylation at imprinted loci (such as histone modifications, miRNAs, and non-imprinted DNA methylation) may be more sensitive to ethanol exposure. Further to this, these dysregulated epigenetic states may be transmitted through the germline, and consequently propagate aberrant epigenetic states during cellular division and embryonic development.

1.6. Project rationale

It is likely that alcohol influences all levels of epigenetic regulation, including DNA methylation, histone modifications and ncRNAs. Chronic exposure of male gametes to alcohol, via oral ingestion, provides a passage through which alcohol can exert an effect on the epigenetic establishment and/or maintenance of epigenetic marks on sperm DNA and sperm-specific miRNAs during spermatogenesis and subsequent sperm maturation.

Spermatozoa are essentially transcriptionally inactive, and the mature sperm genome is highly methylated. Mature mouse sperm DNA is primarily protamine-bound, but 1% of the genome is retained in histone-bound structures. The histone-bound fraction is enriched for regions of developmental importance, and includes promoters of developmental genes,

miRNA clusters, and imprinted gene regions. These regions are generally GC-rich, containing CpG islands and low-methylation regions, which lend themselves to epigenetic regulation via DNA methylation. Promoters of histone-bound developmental genes are generally hypomethylated, and prime the sperm genome for activation during early embryonic development. This study therefore focused on the effect of alcohol on DNA methylation at CpG islands across the sperm genome to explore its role in the effects of preconception paternal alcohol exposure on epigenetic signatures in the male gametes, and subsequent impact on embryonic development.

It was hypothesised that chronic alcohol exposure of male mice prior to conception would alter DNA methylation establishment and/or maintenance at CpG islands contained within developmentally significant genes, during spermatogenesis. Subsequently, these epimutations would be inherited by sired offspring, and consequently dysregulate gene expression, and manifest in a phenotype associated with that observed in foetal alcohol syndrome, including growth restriction, neural deficits and/or craniofacial dysmorphology.

The objective of the study was to determine whether preconception paternal alcohol exposure could induce DNA methylation changes at CpG islands within the sperm epigenome, dysregulate gene expression in embryonic tissues of sired offspring, and elicit an abnormal phenotype.

This study therefore aimed to:

1) Quantify sperm CpG island DNA methylation profiles of male mice chronically exposed to ethanol. Methodology: To quantify sperm DNA methylation, Reduced Representation Bisulfite Sequencing (RRBS) was employed, as it enriches for CpG islands and is able to assess CpG methylation at a single base pair resolution.

2) Determine whether a growth-restriction phenotype was apparent in offspring (embryos) sired by ethanol exposed males. Methodology: Whole embryo, brain, liver and placental weight from day 16.5 embryos (E16.5) derived from alcohol treated males was compared to that of sucrose treated males. E16.5 was chosen, as this is a time at which epigenetic reprogramming of the embryo and its primordial germ cells is complete.

3) Quantify changes in gene expression in embryonic tissues of offspring sired by ethanol treated males. Methodology: A MouseWG-6 v2.0 Expression BeadChip array was used to assess global gene expression profiles in the brain, liver and placenta of E16.5 embryos of ethanol exposed males, and compared to those from embryos sired by sucrose treated males.

The thesis is comprised of four chapters. Chapter 1 provides a literature overview of epigenetic regulatory mechanisms, and the effect of alcohol on these mechanisms. Chapter 2 presents the findings on the effect of chronic alcohol exposure on the sperm epigenome. Chapter 3 presents the findings on the effects of preconception paternal alcohol exposure on offspring outcome, including changes in phenotype and embryonic gene expression. Finally, Chapter 4 presents an overlap analysis of genes which demonstrate both a significant change in sperm DNA methylation and a significant change in embryonic gene expression, and concludes with a section on the epigenetic transmissibility of alcohol-induced epimutations through the male germline, which is associated with abnormal gene expression profiles in offspring, and an altered phenotype.

Chapter 2

The effect of chronic alcohol exposure on methylation signatures of the male gamete

2.1. Introduction

2.1.1. Drinking behaviour of males puts gametes at risk

According to the World Health Organisation (WHO, 2011) the harmful use of alcohol is the leading factor for mortality in men, aged between 15 and 59. The WHO attributes this to injuries, violence and cardiovascular diseases associated with harmful alcohol use – which account for 6.2% of all male deaths globally (compared to 1.1% of females). The total burden of disease in males associated with alcohol use is 7.4%, while it is only 1.4% in females. Globally, approximately 11.5% of drinkers are heavy episodic drinkers (Table 1) (WHO, 2011). However, men exhibit weekly heavy episodic drinking (HED) that is four times higher to that in women, and consistently engage in hazardous drinking at much higher levels in all regions. Of these regions, Africa accounts for the greatest proportion of heavy episodic drinkers (over 25%), and the greatest prevalence of HED amongst men in the world (Table 1). Furthermore, findings by the European School Survey Project on Alcohol and other Drugs (ESPAD) revealed that although there are on average, no gender differences in the frequency of drunkenness among males and females, heavy episodic drinking was more common among boys (15 – 16 years of age) (Hibell et al., 2009). Men also have vastly lower abstinence rates than women. Hazardous and harmful drinking patterns, such as drinking to intoxication and binge drinking,

seem to be on the rise among adolescents and young adults – where its harmful use is the world's leading risk factor for death among males aged 15–59 (as reported in WHO, 2011) – which begins at an early age and continues well into their reproductive years (Figure 19).

Table 1: Prevalence of weekly heavy episodic drinking among drinkers. Measurements were taken over a 12 month period, stratified by WHO region in 2005 (WHO, 2011).

WHO region	Women (%)	Men (%)	Total (%)
AFR	16.2	30.5	25.1
AMR	4.5	17.9	12.0
EMR	17.9	24.9	24.7
EUR	4.6	16.8	11.0
SEAR	12.9	23.0	21.7
WPR	1.3	11.6	8.0
World	4.2	16.1	11.5

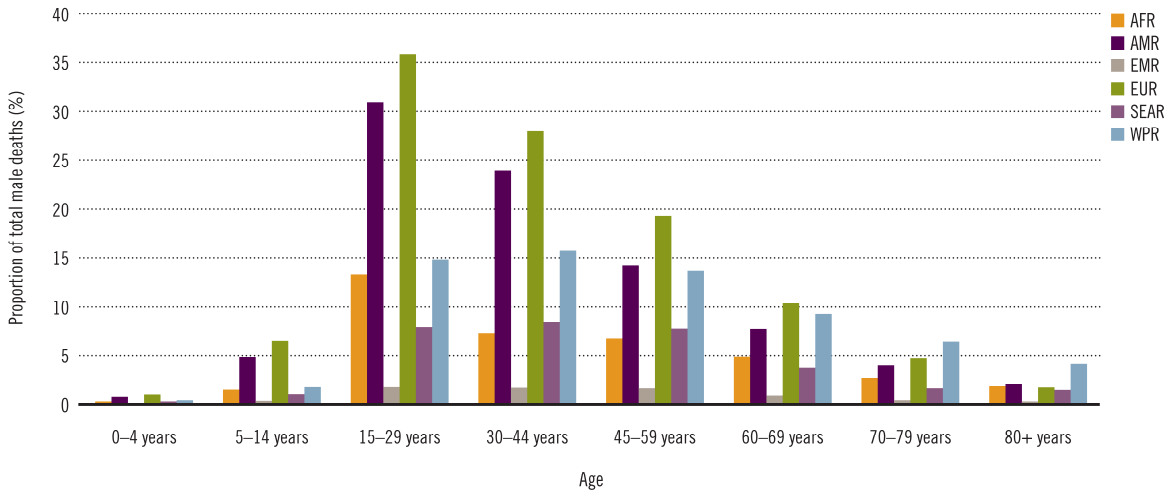


Figure 19: Proportion of alcohol-attributable male deaths (%) of all male deaths by age group and WHO region, 2004. Proportion of total male deaths (%) are shown per age group and WHO region: AFR (Africa), AMR (Americas), EMR (Eastern Mediterranean), EUR (Europe), SEAR (South-East Asia), and WPR (Western Pacific). According to the WHO, alcohol (mis)use is attributed to a large portion of male deaths from age groups 15-29 years old, 30-44 years old, and 45-59 years old. Furthermore, alcohol-related mortalities are most prevalent in the Americas, Europe and Western-Pacific Region (WHO, 2011).

Together, these factors are testament to the risky drinking behaviour that characterises typical male consumption of alcohol on a global level. This of particular concern, as this behaviour and exposure levels to alcohol are at their highest during the male pique reproductive period. This can have serious implications for the development of male gametes, where alcohol exposure has been shown to negatively impact on sperm viability, male fertility and reproductive success (Anderson et al., 1983; Donnelly et al., 1999; Gaur et al., 2010; Muthusami and Chinnaswamy, 2005), as well as sperm DNA integrity (Rahimipour et al., 2013; Robbins et al., 2005).

2.1.2. The effect of alcohol exposure on sperm DNA

The effect of environmental exposures, including toxicants and alcohol, on sperm DNA has been shown to directly impact on DNA integrity (Baccarelli and Bollati, 2009; Barratt et al., 2010; Bielawski et al., 2002; Cortessis et al., 2012; Curley et al., 2011; Delbes et al., 2010; Hou et al., 2012; Maselli et al., 2012), and has also been associated with chromosomal aneuploidies (Kawamura et al., 2005; Robbins et al., 2005). In particular, alcohol exposure has been shown to affect sperm DNA methylation. Alcohol exposure has been shown to affect cytosine methyltransferase mRNA levels (Bielawski et al., 2002), and DNA methylation at imprinted loci, including *H19* and the *IG*-DMR (Knezovich and Ramsay, 2012; Ouko et al., 2009; Stouder et al., 2011).

However, evidence for this effect is limited to a few pre-selected regions, such as imprinted loci, or specific loci where a select few CpG sites act as a proxy for the level of DNA methylation for a particular region. This is problematic as it doesn't give an indication of alcohol's effect at a genome-wide or global level. Studies that globally quantify DNA methylation usually use an average value of the entire epigenome's DNA methylation

content. In this way, DNA methylation is coalesced into a single pooled value. This can be misleading, as opposing effects can be masked. That is, regions that show increased levels of DNA methylation as a result of alcohol exposure, are nullified by regions that show decreased levels when they are added together.

Thus, comprehensive evidence is lacking about genome-wide, site-specific changes in DNA methylation in relation to ethanol exposure of mature male sperm. The current study therefore utilised RRBS to explore the effects of ethanol exposure at the level of individual CpG sites contained within the CpG island enriched portion of the epigenome in a mouse model.

2.2. Materials and Methodology

To test the effect of preconception alcohol exposure on DNA methylation profiles in mature male mouse gametes on a genome-wide level, a C57BL/6 mouse model was established with two mouse models. In the first mouse model, sperm was collected from male mice chronically exposed to either alcohol or sucrose (control mice) for 27 weeks. In the second model (essentially the same in design), sperm was collected from male mice chronically exposed to either alcohol or sucrose for 10 weeks. However, in the second mouse model offspring (embryos) from the treated (ethanol or sucrose) males were also investigated.

2.2.1. Paternal ethanol exposure models

Two mouse models were established to test the effect of ethanol exposure on DNA methylation in the male gametes. The first model was setup at the Central Animal Service, University of the Witwatersrand, South Africa ("SA model"). The second model was subsequently established (after the first model was completed) at the Department of Physiology, Development and Neuroscience, University of Cambridge, United Kingdom ("CAM model"). Both of the mouse models (SA and CAM) made use of C57BL/6 mice originally obtained from the Jackson Laboratory, and an ethanol exposure dosage of 7.5µl/g of a 50% solution of pharmaceutical grade ethanol (2.96g/kg). This dosage has been shown to simulate a pharmacologically significant effect (Rhodes et al., 2005), which results in a blood alcohol concentration of approximately 1.0 mg/ml. Similarly, control male mice in both models were exposed to an isocaloric solution of a 0.704g/ml sucrose solution at 7.5µl/g (which equates to approximately 2,700 calories – equal to that derived from the alcohol intake in the ethanol-treated males). The SA model consisted of fifteen male mice in each treatment group (ethanol and sucrose control), while the CAM model consisted of 10 male mice per group. Food and water was given *ad libitum*. Oral gavaging was carried out over a 27 and 10 week period (SA and CAM models), respectively.

2.2.2. Blood alcohol concentration quantitation

In order to ascertain whether a pharmacologically significant blood alcohol concentration (BAC) was achieved by administrating alcohol/ethanol at 2.96g/kg, BAC measurements were assessed using the BioVision Ethanol Assay Kit (BioVision Research Products, CA, USA). Briefly, saphenous vein blood was obtained from male mice 30min post-gavage with either 2.96g/kg of ethanol (n=6) or 7.5µl/g of 0.704g/ml sucrose solution (n=5) in heparinised

capillary tubes. This period was chosen as it has been demonstrated that peak BAC occurred at this time point regardless of ethanol dosage (Bielawski and Abel, 2002). Serum was obtained by centrifugation of whole blood using Micron serum filters (Merck Millipore, MA, USA) for 20min at 10,000 rpm. Serum was diluted to 1:200 with the Ethanol Assay Buffer. The colorimetric assay was employed to quantify BACs of both ethanol exposed (ETOH) and control (CTRL sucrose exposed) mice.

2.2.3. Sperm DNA extraction

Sperm DNA was obtained from males from both mouse models, wherein males were chronically exposed to either ethanol or sucrose prior to harvesting sperm.

In the SA model, sperm was harvested immediately following the 27 week exposure regimen. In the CAM model (where the male mice needed to sire offspring), sperm was harvested from treated sires within a seven day period following confirmation of a vaginal plug in untreated females with whom they were mated (ethanol or sucrose treatment was continued through the seven day period).

In both mouse models, mature spermatozoa were harvested by epididymal dissection, followed by a swim-out protocol (Appendix A). Sperm DNA was extracted using the QIAGEN Supplementary Protocol: *Purification of DNA from epithelial cells mixed with sperm cells using the QIAamp® DNA Micro Kit* (QIAGEN, Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's specifications. Steps 1 – 14 were replaced with the protocol supplied in Appendix B.

2.2.4. Quantitation of sperm DNA methylation levels

2.2.4.1. Locus-specific quantitative pyrosequencing of paternal ICRs

In order to assess the effect of chronic alcohol exposure on the methylation status of the three paternally methylated imprinting control regions (*Rasgrf1*, *IG-DMR*, and *H19*), locus-specific quantitative pyrosequencing was conducted on sperm DNA obtained from the SA model (fifteen sperm DNA samples from each treatment group).

Bisulfite conversion

Approximately 100ng of sperm DNA was bisulfite modified using the Sigma Imprint® DNA Modification Kit (Sigma-Aldrich Company Ltd., Saint Louis, Missouri, USA), using the Two-Step Modification Procedure as per the manufacturers protocol. Briefly, double-stranded DNA is denatured to single-stranded DNA, which allows bisulfite to specifically deaminate cytosine bases into uracil residues. Converted DNA is then passed through a filter column with high DNA binding affinity, allowing for effective removal of excess sodium bisulfite and salts from each sample. Membrane-bound, bisulfite converted DNA was eluted in 15µl of Elution Buffer.

PCR amplification of paternal ICRs

Pyrosequencing assays were designed using the Pyro Q-CpG™ software (QIAGEN, Hilden, North Rhine-Westphalia, Germany). ICRs were PCR amplified using Bio-Rad DNA Engine® Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, California, USA) according to Table 2.

Table 2: Pyrosequencing PCR conditions

Reagent	Volume	Cycle conditions		
10x PCR Buffer (containing $MgCl_2$)	2.5µl			
dNTP mix (10mM)	1µl	95˚C	15 min	
HotStar Taq (QIAGEN)	0.125µl	94˚C	30 sec	
Bisulfite converted DNA	1µl	Ta˚C*	30 sec	40 cycles
Primer F (10µM)	0.5µl	72˚C	30 sec	
Primer R (10µM)	0.5µl	72˚C	10 min	
ddH2O	19.375µl			
	25µl			

* Annealing temperatures (Ta°C) and primer sequences for the amplification of the ICRs from bisulfite modified DNA are recorded in Table 3

Locus-specific quantitative pyrosequencing

Allele-specific quantitative pyrosequencing was performed in duplicate using the PyroMark Q96 MD and analysed using the Pyro Q-CpG™ software (QIAGEN, Hilden, North Rhine-Westphalia, Germany). Sequencing (S) primers were designed for each assay (Table 3). Each assay included a non-CpG cytosine as an internal control for bisulfite conversion, and samples with >5% methylation at this position were excluded from further analyses.

A 191bp region of the *paternally expressed gene 3* (*Peg3*-DMR), containing 5 CpG sites in the pyrosequencing assay, was included as a control for the detection of somatic contamination in sperm DNA samples. *Peg3* is a maternally imprinted gene contained within the proximal domain on mouse chromosome 7 (Kuroiwa et al., 1996), and therefore expected to be unmethylated on the paternal allele. Thus, detection of significant levels of methylation (>5%) at this region would be indicative of somatic cell contamination (i.e. the presence of the hypermethylated maternal allele).

Table 3: Pyrosequencing assay details

Region Primer	Primer sequence (5' – 3')	PCR amplicon size (bp)	Ta (°C)
IG-DMR*			
IG-DMR-F	GTGGTTTGTTATGGGTAAGTTT	318	54
IG-DMR-R	CCCTTCCCTCACTCCAAAAATTAA		
IG-DMR-S	TGGTTTATTGTATATAATGT		
H19-CTCF2*			
H19-DMR-F	GGGGGGTAGGATATATGTATTTTT	226	54
H19-DMR-R	ACCTCATAAAACCCATAACTATAAAATCAT		
H19-DMR-S	GTGTGTAAAGATTAGGG		
Rasgrf1-DMR			
Rasgrf1-DMR-F	GGGAAGATTATTAGTTGGGGAGGTG	177	54
Rasgrf1-DMR-R	CAACAAAAACCAAAATATCAATCCTAAC		
Rasgrf1-DMR-S	ATTAGAGTTAAATATAAAGAATGG		
Peg3-DMR*			
Peg3-DMR-F	TTGGATTGGTTAGAGAGGAAGT	191	60
Peg3-DMR-R	ACAATCTAATACACCCACACTAA		
Peg3-DMR-S	GGAGAGATGTTTATTTTG		

*sequences obtained from Sun et al. (Sun et al., 2012)

Statistical analyses

A Mann-Whitney U Test was used to determine DNA methylation differences between the two treatment groups at the level of individual CpG sites within each of the three paternal ICRs (*Rasgrf1*, *IG-DMR*, and *H19*). Statistical analyses were performed using Statistica 10 (StatSoft. Inc., Tulsa, Oklahoma, USA).

2.2.4.2. Reduced representation genome-wide quantitative sequencing

In order to assess the effect of chronic alcohol exposure at regions in the genome which rely heavily on DNA methylation for the regulation of associated genes, Reduced Representation

Bisulfite Sequencing (RRBS) was employed to quantitatively sequence regions of the epigenome that are enriched for CpG islands. RRBS data was generated for both the SA and CAM models.

The RRBS technique employs a strategy that generates short fragments, representative of approximately 1% of the genome, but which enriches for densely populated CpG sites within it (Meissner et al., 2008). These include CpG islands contained within promoter and enhancer regions, and gene bodies of actively transcribed genes (Laurent et al., 2010).

Briefly, RRBS makes use of the methylation insensitive enzyme, *MspI*, to generate small genomic fragments of approximately 36 bases flanked by CpG sites. This is achieved due to the fact that *MspI* cuts outside of the CG dinucleotide contained with its CCGG palindromic recognition site (i.e. (C↓CGG)/(GGC↑C)). Thus, each fragment and sequencing read covers at least one informative CpG (which is either methylated or unmethylated). This enrichment step consequently reduces the number of reads required to obtain a high coverage of a reproducible fraction of CpGs (Gu et al., 2011). Following DNA isolation and digestion with *MspI*, the 3'-terminal overhangs of each fragment are filled with an adenosine (End Repair with A-tailing) – which is required for subsequent adapter ligation. Following adapter ligation, genomic fragments are size-selected by extracting 40 – 220bp fragments from an agarose gel. The fragments are subsequently bisulfite modified, and amplified via PCR to produce a final library for RRBS.

RRBS of sperm from chronically exposed male mice

The RRBS protocol used in this study was obtained via personal communication with Dr Sebastien Smallwood (Babraham Institute, Cambridge, UK), and adapted (Appendix C). Briefly, approximately 10ng of sperm DNA (extraction described in Section 2.2.3) was used to generate each RRBS library, as follows: (1) DNA was digested overnight using the

methylation-insensitive enzyme, *Msp*I (2) End Repair / A-Tailing (3) Adapter ligation (4) Bisulfite modification (5) First amplification step (6) Fragment size selection (7) Second amplification step (8) Purification (AMPure XP magnetic beads) (9) QC analysis (10) Library quantification and sequencing.

In order to reduce sequencing costs, while still capturing representative variation within each treatment group, sperm DNA samples were pooled. Each pool contained sperm DNA from five individual males from the same treatment group (either ethanol-treated males or sucrose-treated males) and the same model (either SA or CAM). Five pools were created for each treatment group – three from the SA model (total pooled sperm DNA samples = 15) and two from the CAM model (total pooled sperm DNA samples = 10). Thus, a total of 10 sperm DNA pools (representative of 50 individual sperm DNA samples) were generated for RRBS (three ethanol-treated from the SA model, two from the CAM model; and three sucrose-treated from the SA model, two from the CAM model).

In order to obtain an equimolar contribution from each of the five sperm DNA samples that comprised each pool, it was essential to accurately quantify the concentration of each individual sperm DNA sample prior to pooling. Initial/crude concentrations were determined using a spectrophotometer, and samples were diluted to approximately 5ng/µl in ddH_2O. Subsequently, concentrations were accurately measured using the Quant-iT™ PicoGreen® (Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. Briefly, a standard curve of lamda DNA was diluted from 1µg/µl to 1ng/µl and all samples diluted 1/50. To 50µL of DNA sample was added 50µl of 1x Quant-iT™ PicoGreen®. All samples and the standard curve were assessed in triplicate. Samples were assayed in 96 well microplates using a FLUOstar OPTIMA (BMG LabTech, Ortenberg, Germany) microplate reader. DNA content in each sample was quantified at an excitation frequency of 485nm and emission frequency of 520nm. Concentration of the samples extrapolated from the standard

curve. An equal quantity of each of the five DNA samples was then used to obtain a total of 10ng for each pool, which was used for RRBS library preparation.

RRBS next-generation sequencing data was generated for the 10 sperm DNA pools using an Illumina Genome Analyzer II (Illimina Inc., San Diego, California, USA) at the Babraham Institute (Cambridge, UK).

Bioinformatics approach

The bioinformatics approached employed in the current study initially filtered for informative CpG sites that contained a sufficient read depth. A sliding window approach was then used to allocate informative CpG sites into 500bp bins. Adjacent bins that contained informative CpG sites, were then grouped into a single region. The average DNA methylation was quantified for each region, and each region was allocated to its nearest gene. Average DNA methylation was then calculated across the regions that were allocated to a single gene, and thus, a single average DNA methylation level was calculated for each gene.

Stringent quality control of raw sequence reads was achieved using TrimGalore and Cutadapt to remove adaptor sequences and nucleotide bases with poor qualities. Sequence alignments were performed using Bismark against the mouse reference genome assembly NCBI37/mm9. No de-duplication was used, as it is not suitable for RRBS data.

Methylation levels for each CpG cytosine (on both sense and anti-sense strands) was calculated using Bismark methylation extractor and custom Perl script. Rigorous quality control steps were performed as follows:

QC Step 1: CpG dinucleotides with less than 5 mapped reads were removed from further analysis and marked as uninformative.

QC Step 2 (within group QC): in order to assess whether there was a high degree of correlation between the RRBS datasets, a Pearson correlation was performed. This correlation was used to identify CpG sites with DNA methylation levels that did not deviate more than 20% between samples (RRBS sperm pools) within the same treatment group (ETOH and sucrose/CTRL). CpG sites that showed DNA methylation levels that deviated by >20% in a given pool when compared to the other pools in the same treatment group, were removed from further analyses.

QC Step 3 (between group QC): a CpG dinucleotide which exists only in the ETOH data but not in any of CTRL data (or vice versa), which renders them incomparable, was removed from further analysis.

To assess regions within the epigenome that demonstrated changes in sperm DNA methylation levels in the ethanol-treated group (when compared to the sucrose-treated control group) a sliding-window algorithm with a 500bp window and 200bp stride was used. 500bp windows with no informative CpG dinucleotides captured were removed from further analysis. Adjacent overlapping 500bp windows were clustered together if they contained at least one informative CpG dinucleotide.

The comparison strategy employed to determine significant differences in DNA methylation between the ethanol-treated and control (sucrose-treated) groups in the SA model, is summarised in (Figure 21). In order to obtain a single control dataset, representing the control (assumed to be “normal” or “wild-type”) level of DNA methylation to which the ethanol-treated data could be compared, the three SA model control pools were merged into one using a systematic approach outlined in (Figure 20). Briefly, this approach minimised the potential for the introduction of bias (or skewing of data point values) in

instances where a data point (level of DNA methylation at a given CpG site) in one pool is considerably different to that same data point in the other control pools. A similar strategy was employed for the CAM model (schematic outline not shown). These single control DNA methylation datasets were termed "CTRL-SA" (single control dataset obtained from the SA model) and "CTRL-CAM" (single control dataset obtained from the CAM model).

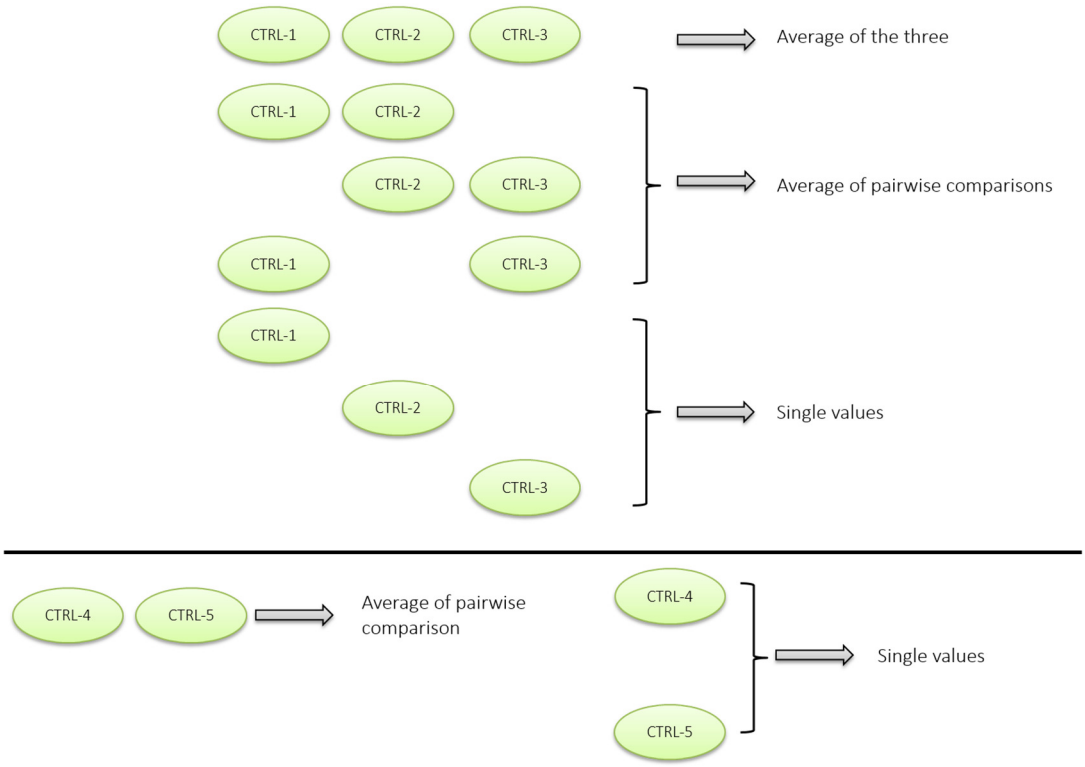


Figure 20: Approach to merge SA model control sperm DNA methylation data. The SA model consisted of three control groups (CTRL-1, -2, and -3), with each comprised of six pooled sperm DNA samples from sucrose exposed mice. In order to obtain a single control sample profile (normal sperm DNA methylation profile to compare against that of alcohol exposed samples), (1) DNA methylation values at each informative CpG site within the three control groups were averaged (2) Each control sample was compared to each other control sample, and an average of each pair was obtained (3) Each control sample's individual DNA methylation value was otained. The average DNA methylation value of comparisons (1), (2) and (3) was then used as the single control DNA methylation level for each CpG site (CTRL-SA). A similar startegy was used for the CAM model. Unlike the SA model, the CAM model consisted of two control groups (CTRL-4 and -5), with each comprised of six pooled sperm DNA samples from sucrose exposed mice. The average of the average DNA methlayion of the two samples and each sample's resepctive average DNA methylation was used as the single control DNA methylation level for each informative CpG site (CTRL-CAM).

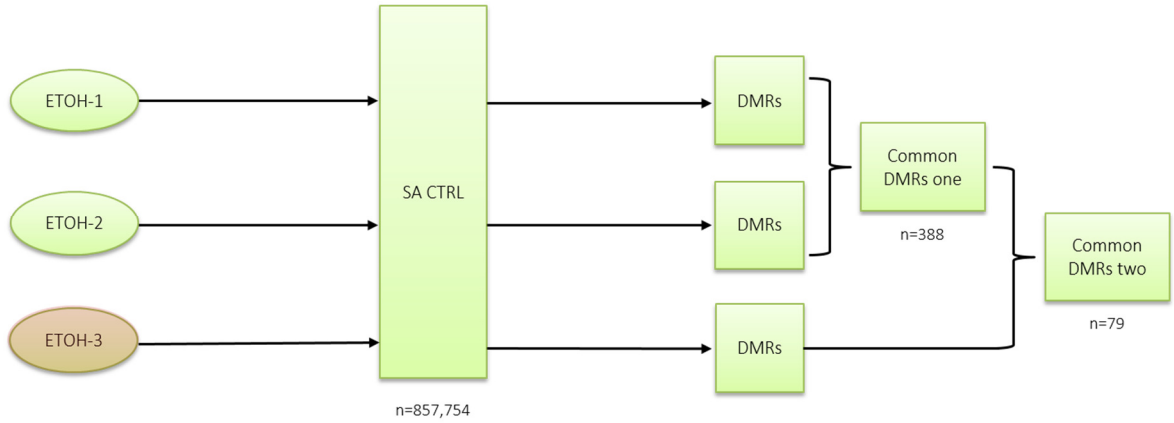


Figure 21: Comparison strategy to assess DNA methylation differences between the ethanol and control sperm samples (pools). Depicted is the comparison strategy used to compare DNA methylation levels of each ETOH sample to the single averaged control sample obtained for the SA model (as described in Figure 20) which yielded 857,754 informative CpG sites (CAM model not shown). Each ETOH sample was compared against the single averaged control sample to identify DMRs common to both samples, and the difference in DNA methylation between the two samples was claculated. DMRs common to both ETOH-1 v. CTRL-SA and ETOH-2 v. CTRL-SA were first identified (Common DMRs one), and then those common to ETOH-3 v. CTRL-SA and Common DMRs one were identified (Common DMRs two). The data-limiting sample, ETOH-3 is highlighted in orange.

2.3. Results

2.3.1. Chronic alcohol exposure has nuanced effects on sperm paternal ICR methylation

Physiologically significant blood alcohol concentration

In order to test whether physiologically significant blood alcohol concentrations (BACs at 1mg/ml) were achieved using our alcohol regimen, five ethanol exposed and six sucrose exposed mice were used to assess BAC levels. Physiologically significant blood alcohol concentrations of 1.15 ± 0.45mg/ml were achieved in male mice exposed to approximately 3g/kg of ethanol when compared to that of control mice (0.48 ± 0.17mg/ml; Mann Whitney one-tailed test p=0.0002) (Table 4).

Table 4: Blood alcohol concentrations of exposed male mice.

Treatment	BAC (mg/ml)
EtOH 1	0.947
EtOH 2	1.262
EtOH 3	0.912
EtOH 4	1.897
EtOH 5	0.752
Control 1	0.413
Control 2	0.479
Control 3	0.507
Control 4	0.727
Control 5	0.550
Control 6	0.216

Locus-specific ICR CpG methylation

Locus-specific sperm DNA methylation was quantified in the paternal imprinting control regions (ICRs) of male mice from the SA model. Chronic exposure of male mice to approximately 3g/kg of ethanol for a period of 27 weeks revealed a general trend for decreased ICR methylation in sperm DNA when compared to the control (sucrose-treated) group of sires (Figure 22). Significant site-specific reduced methylation was noted at only a single CpG site within the *IG*-DMR (CpG 2, DNA methylation change=1.33%, p=0.031) (Table 5).

Table 5: Quantitative pyrosequencing data of mouse sperm ICR methylation from treated sires.

Region	Ethanol-treated males		Sucrose-treated males		*p*-Value
	Mean Methylation (%) ± Std Dev	n	Mean Methylation (%) ± Std Dev	n	
IG_DMR					
IG-DMR CpG 1	98.03 ± 1.793	16	98.75 ±1.516	14	0.120
IG-DMR CpG 2	95.78 ± 1.505	16	97.11 ±1.546	14	0.031*
IG-DMR CpG 3	98.53 ± 1.802	16	98.18 ±1.564	14	0.355
IG-DMR CpG 4	90.22 ± 1.798	16	90.96 ±2.135	14	0.313
IG-DMR CpG 5	99.91 ± 0.272	16	99.54 ±1.232	14	0.637
IG-DMR CpG 6	99.81 ± 0.629	16	100.00 ± 0.000	14	0.580
IG-DMR CpG 7	98.28 ± 1.712	16	98.79 ± 1.236	14	0.608
H19 ICR					
H19 CpG 1	99.53 ± 0.562	16	99.50 ± 1.193	14	0.448
H19 CpG 2	95.94 ± 1.031	16	96.57 ± 0.958	14	0.085
H19 CpG 3	95.13 ± 1.103	16	95.43 ± 0.938	14	0.313
H19 CpG 4	98.16 ± 1.535	16	97.82 ± 1.957	14	0.790
H19 CpG 5	99.53 ± 0.884	16	99.46 ± 0.771	14	0.637
H19 CpG 6	93.91 ± 1.020	16	94.00 ± 1.710	14	0.498
Rasgrf1 ICR					
Rasgrf1 CpG 1	86.97 ± 2.513	16	87.18 ± 2.006	14	0.854
Rasgrf1 CpG 2	94.03 ± 1.396	16	94.36 ± 1.406	14	0.498
Rasgrf1 CpG 3	80.78 ± 2.049	16	80.21 ± 4.228	14	0.294
Rasgrf1 CpG 4	86.22 ± 2.316	16	84.39 ± 3.083	14	0.093

*$p<0.05$

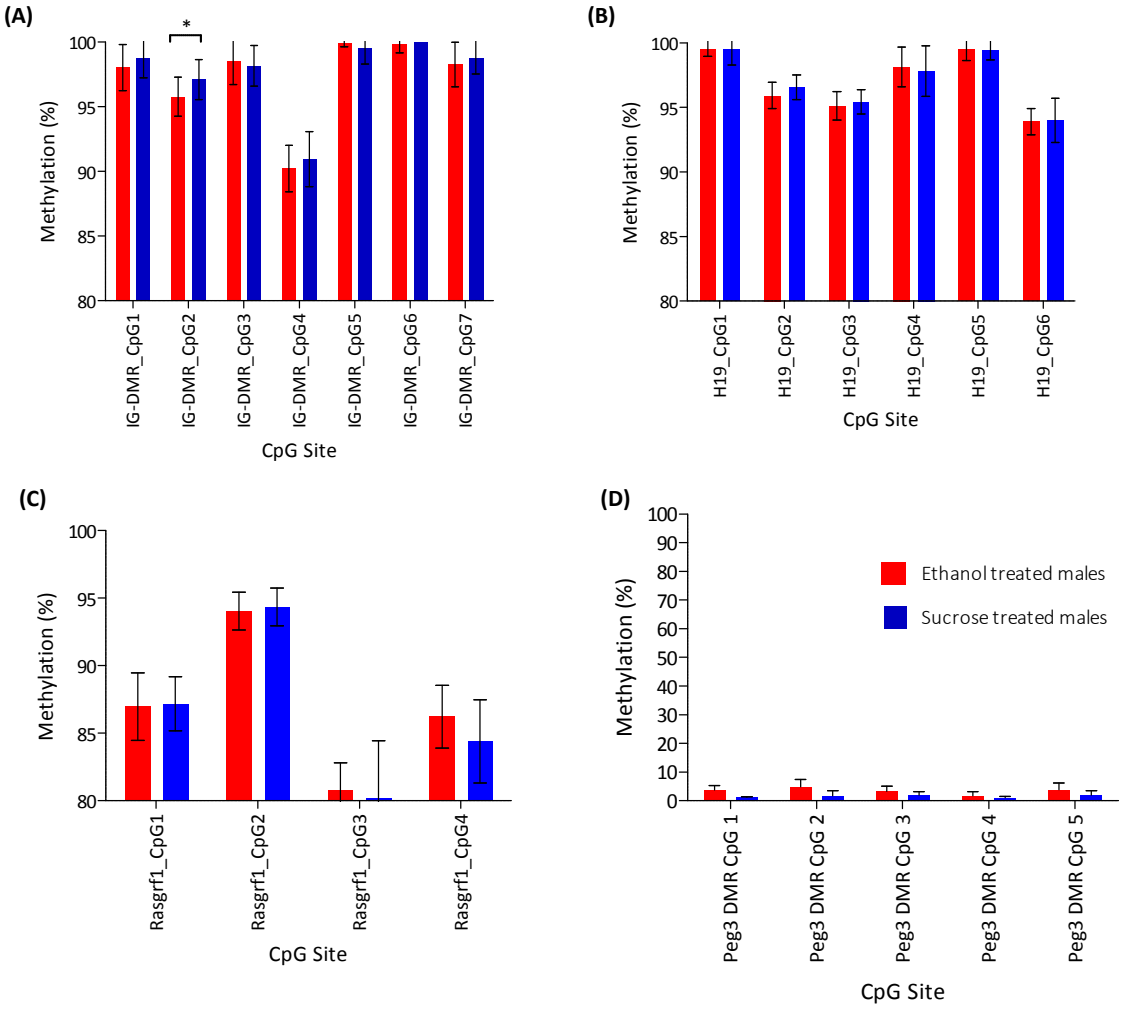


Figure 22: ICR DNA methylation of alcohol exposed mice from the SA model. Graphical comparison of DNA methylation levels at individual CpG sites contained within the *IG*-DMR (A) *H19* ICR (B) *Rasgrf1* DMR (C) and *Peg3* DMR (D). *IG*, *H19* and *Rasgrf1* are paternal ICRs, and are generally hypermethylated in the sperm. The *Peg3* DMR is a maternal ICR, and is therefore hypomethylated in the sperm. Only IG-DMR CpG2 site 2 was significantly differentially methylated ($p<0.05$) in ethanol exposed sperm when compared to sucrose exposed sperm.

2.3.2. Chronic alcohol exposure disrupts methylation profiles across the sperm methylome

To assess the effect of chronic alcohol exposure on male gamete DNA methylation at a single-base resolution, the six sperm DNA pools from the SA mouse model and the four DNA pools from the CAM model (representative of ethanol-treated and sucrose-treated male mice) were used for RRBS analysis to quantify genome-wide levels of DNA methylation. Investigations into the distribution of DNA methylation across sperm methylomes revealed that the vast majority (approximately 50%) of CpG sites showed DNA methylation of between 0 and 10%, while only approximately 20% of the sperm genome was hypermethylated (90-100%) (Figure 23).

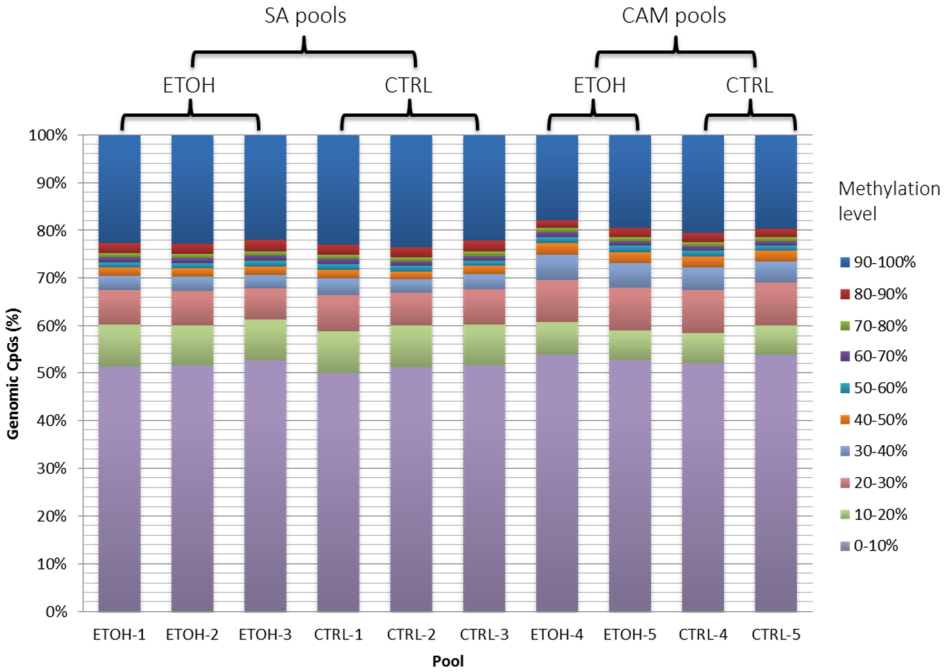


Figure 23: DNA methylation distribution across all sperm pools. The distributions of DNA methylation obtained from RRBS pools of the sperm genome across all loci are divided into 10% bins (shown in the key on the right). Our data demonstrate that a large portion (~50%) of the sperm genome captured by RRBS (CpG islands) is hypomethylated (0-10%) across ETOH and CTRL pools, while only ~20% is hypermethylated (90-100%). This is in contrast to genome-wide methylation of the sperm genome, which is generally hypermethylated.

2.3.2.1. RRBS data filtering and quality control

A summary of the mapping efficiencies of respective specific RRBS libraries are shown in Table 6. All data were analysed using the Illumina 1.5 pipeline, using single end reads and 40bp fragments. Each sperm DNA pool consisted of five independent sperm DNA samples. The SA model made use of three ethanol-treated (EtOH) DNA pools (male mice chronically exposed to alcohol, pools EtOH-1, -2, and -3) and three control (CTRL) DNA pools (male mice exposed to an isocaloric dose of sucrose, pools CTRL-1, -2, -3). Similarly, the CAM model made use of two ethanol pools (EtOH-4 and -5) and two control pools (CTRL-4 and -5). On average, 29 million raw single end reads were obtained from each pool. However, pool EtOH-3 only produced approximately 16 million raw reads. This occurred as the result of a technical issue during the sequencing run. Pool EtOH-3 thus became the data-limiting dataset in further analyses.

The number of uniquely mapped reads was then determined for each sperm pool (Table 6). These reads are representative of the number of unique 40bp fragments sequenced across the RRBS-captured genome (redundant 40bp reads were collapsed into a single 40bp region). On average, approximately 19 million uniquely mapped reads were obtained for all sperm pools, except for pool EtOH-3, which only captured approximately 5 million uniquely mapped reads. Mapping rates are representative of the percentage of raw reads that mapped to unique regions (uniquely mapped reads). On average, these rates were approximately 65% for all pools, except for the data-limited pool (EtOH-3), which had a mapping rate of 34.8%.

Table 6: Mapping efficiency data of RRBS.

Model	Pool ID	Male Treatment	Raw reads (Single ended)	Processed reads	Uniquely mapped reads	Mapping rate
SA	EtOH-1	EtOH	28,456,852	27,948,817	18,871,621	67.5%
	EtOH-2	EtOH	29,214,949	28,744,144	18,966,192	66.0%
	EtOH-3	EtOH	15,971,179	14,200,832	4,945,626	34.8%
	CTRL-1	Sucrose	29,010,729	28,511,158	19,068,038	66.9%
	CTRL-2	Sucrose	29,801,541	29,260,235	19,716,435	67.4%
	CTRL-3	Sucrose	27,604,058	27,149,569	17,703,883	65.2%
	EtOH-4	EtOH	31,753,099	30,966,024	19,927,034	64.4%
	EtOH-5	EtOH	31,847,179	31,161,154	20,210,949	64.9%
	CTRL-4	Sucrose	32,615,837	31,883,047	21,278,465	66.7%
	CTRL-5	Sucrose	29,949,224	29,284,524	19,503,739	66.6%

Following the identification of uniquely mapped reads, the number of CpG sites that were captured in the uniquely mapped reads was calculated for each pool of each model (first row in Figure 24 and Figure 25). On average, approximately 1.2 million unique CpG sites were captured in each pool.

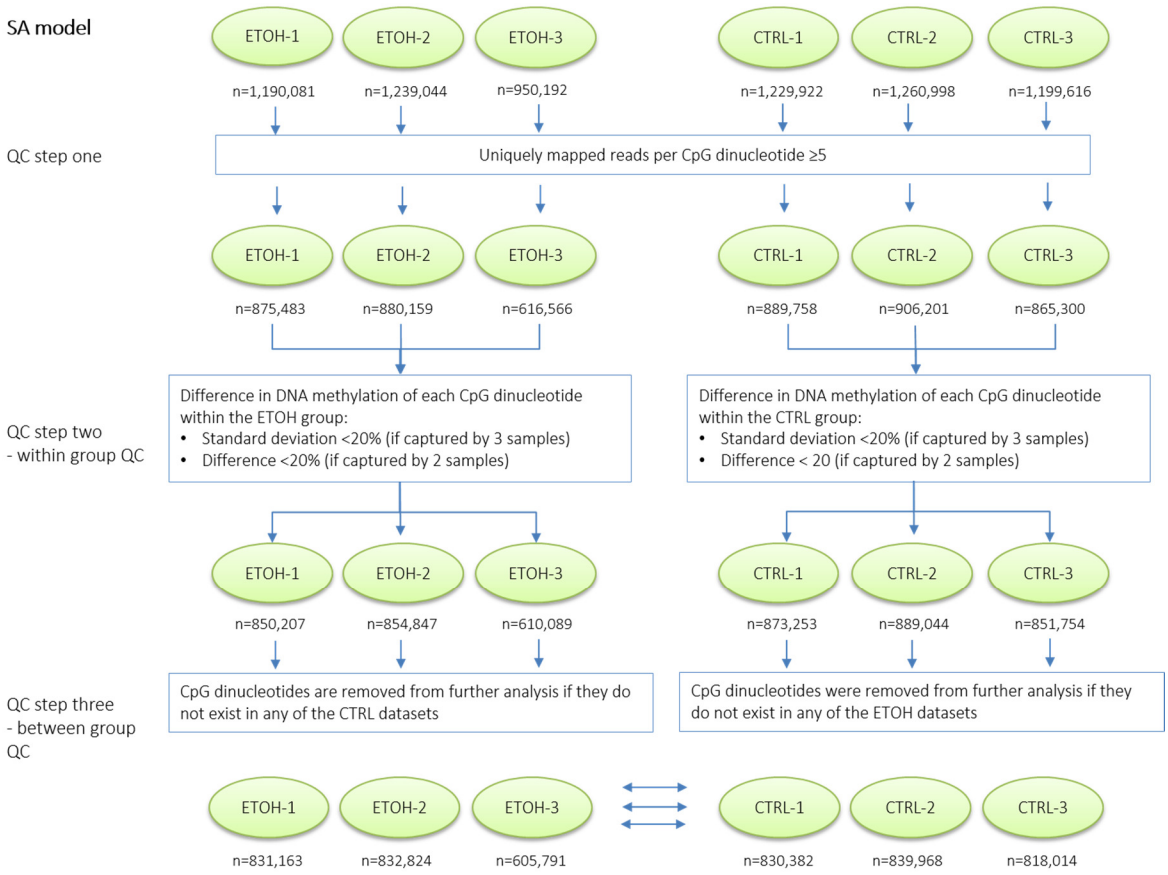


Figure 24: An overview of the quality control process for the SA model RRBS DNA methylation data.

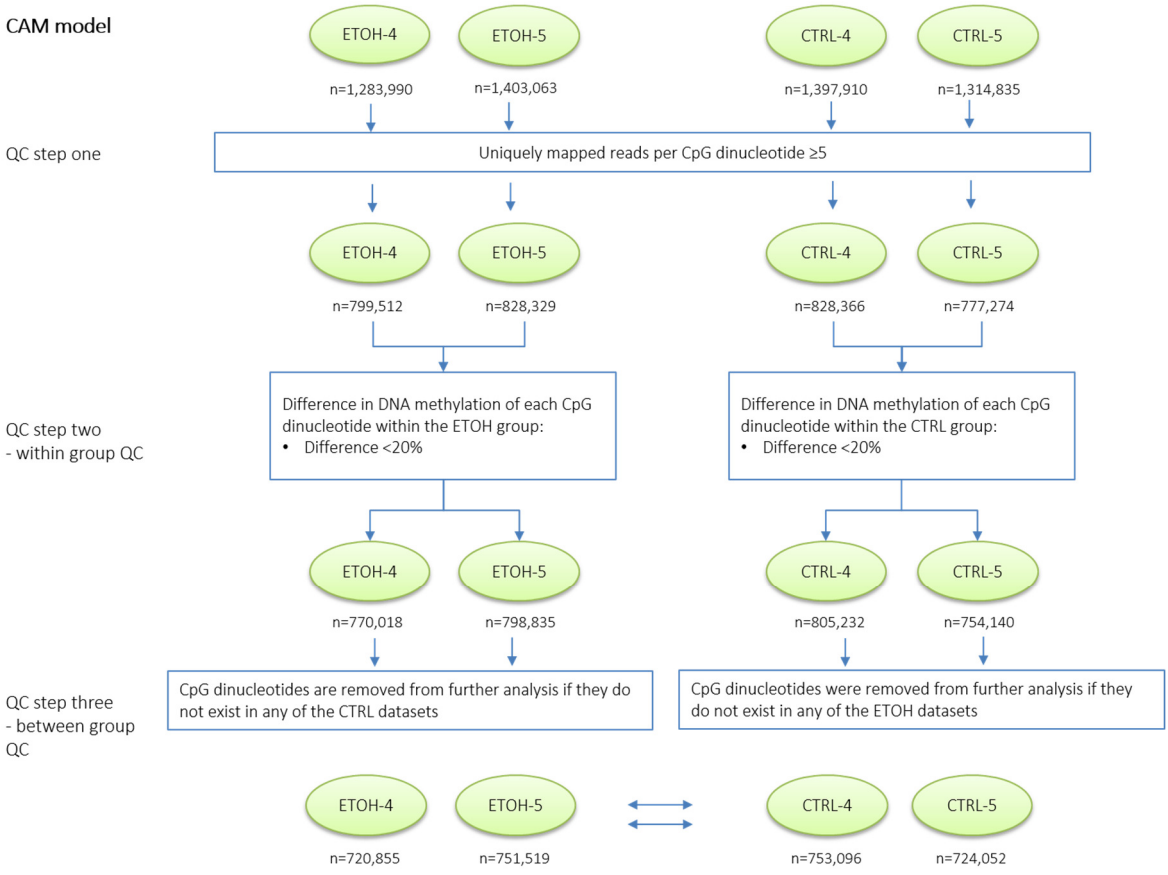
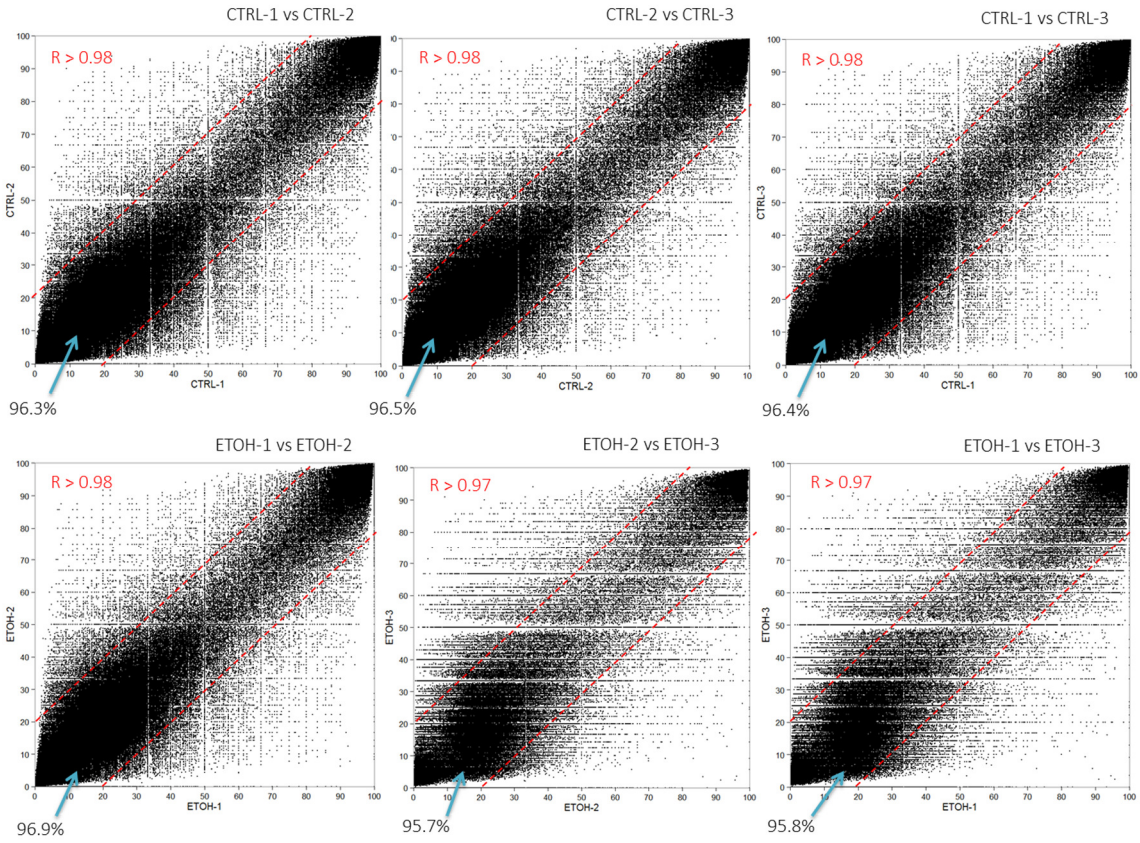


Figure 25: An overview of the quality control process for the CAM model RRBS DNA methylation data.

The first quality control step filtered for CpG dinucleotides with ≥5 mapped reads ("informative" CpG dinucleotide sites). This was important, as a read depth of ≥5 is required in order to give confidence about the methylation status of each CpG site, as well as to quantify the level of methylation at each dinucleotide. On average, each pool had approximately 880,000 and 800,000 informative CpG sites in the SA model and CAM model, respectively, while there were only 616,566 informative CpGs in pool EtOH-3 (Figure 24 and Figure 25). Informative CpG sites were used for all subsequent analyses.

For the second quality control step, a Pearson's correlation was performed in order to determine how well the control replicates from the SA model (CTRL-1, -2 and -3) and CAM model (CTRL-4 and -5) correlated (Figure 26). Similarly, ethanol pools were compared within each model. A 20% cut-off was used, which captured >95% of CpG sites (R>0.97).

SA model



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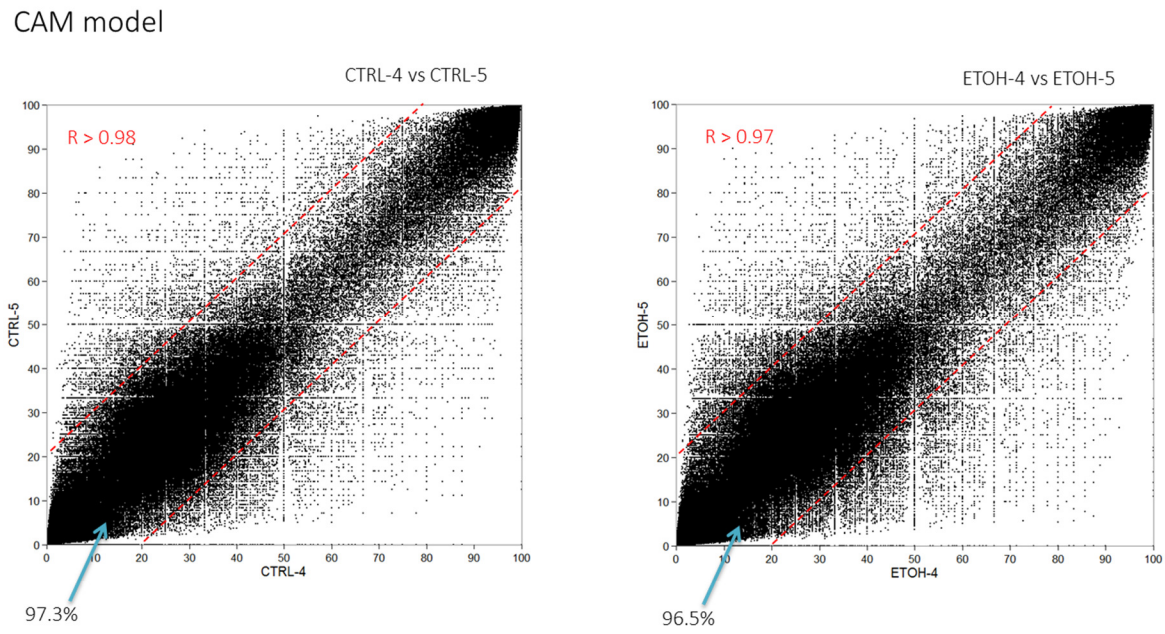


Figure 26: RRBS data correlation of control sperm pools. Blue arrows indicate the percentage of CpG sites captured within the 20% deviation in DNA methylation cut-off (red dashed lines). R value denotes Pearson's correlation coefficient.

It was observed that the control pools and ethanol pools were highly correlated, with a Pearson correlation coefficient of >0.97 across all pools. Furthermore, the vast majority of the data (over 96% of all informative CpG sites) fell within a 20% deviation (denoted by the diagonal area demarked by two dashed red lines in Figure 26). Thus the entire group of controls demonstrated a high level of correlation between one another (within group correlation). CpG sites that fell outside of this demarked area of deviation were removed from further analyses (as these CpG sites demonstrated a high variability in DNA methylation levels within the control group). Similarly, within group variation (deviation) was calculated for the ethanol group, where CpG sites with greater than 20% variation were removed from further analyses.

The second quality control step resulted in approximately 850,000 and 770,000 informative CpG sites from the SA and CAM models, respectively, with sufficient read depth (≥5) and

within group variation of <20% (610,089 CpG sites for pool EtOH-3) (third row in Figure 24 and Figure 25).

The third quality control step determined informative CpG sites that were common to both the control and ethanol groups. Identifying informative CpG sites common to both treatment groups enables the comparison of the DNA methylation level of a given CpG site within the control group with that of the ethanol exposed group. By quantifying the DNA methylation level at a given CpG site in both groups, differences in DNA methylation can be determined. On average, each pool contained 830,000 and 740,000 common CpG sites within the SA and CAM models, respectively (with pool ETOH-3 only having 605,791 common CpG sites) (last step in Figure 24 and Figure 25). Uncommon CpG sites were removed from further analyses.

2.3.2.2. Sperm DNA methylation regions significantly altered by chronic alcohol exposure

Identification of differentially methylated regions

To identify genetic regions (CpG sites) in the ethanol pools that deviated significantly from the control levels of DNA methylation (differentially methylated regions (DMRs)), a binomial p-value threshold of 0.01 was included for the SA model, while $p<0.001$ was used for the CAM model (Figure 27). A higher *p*-value cut-off was used for the CAM model was used in an attempt to reduce the number of significant genes identified. This model would have yielded as vast number of genes with a significance cut-off of $p<0.01$ as only two biological replicates were used.

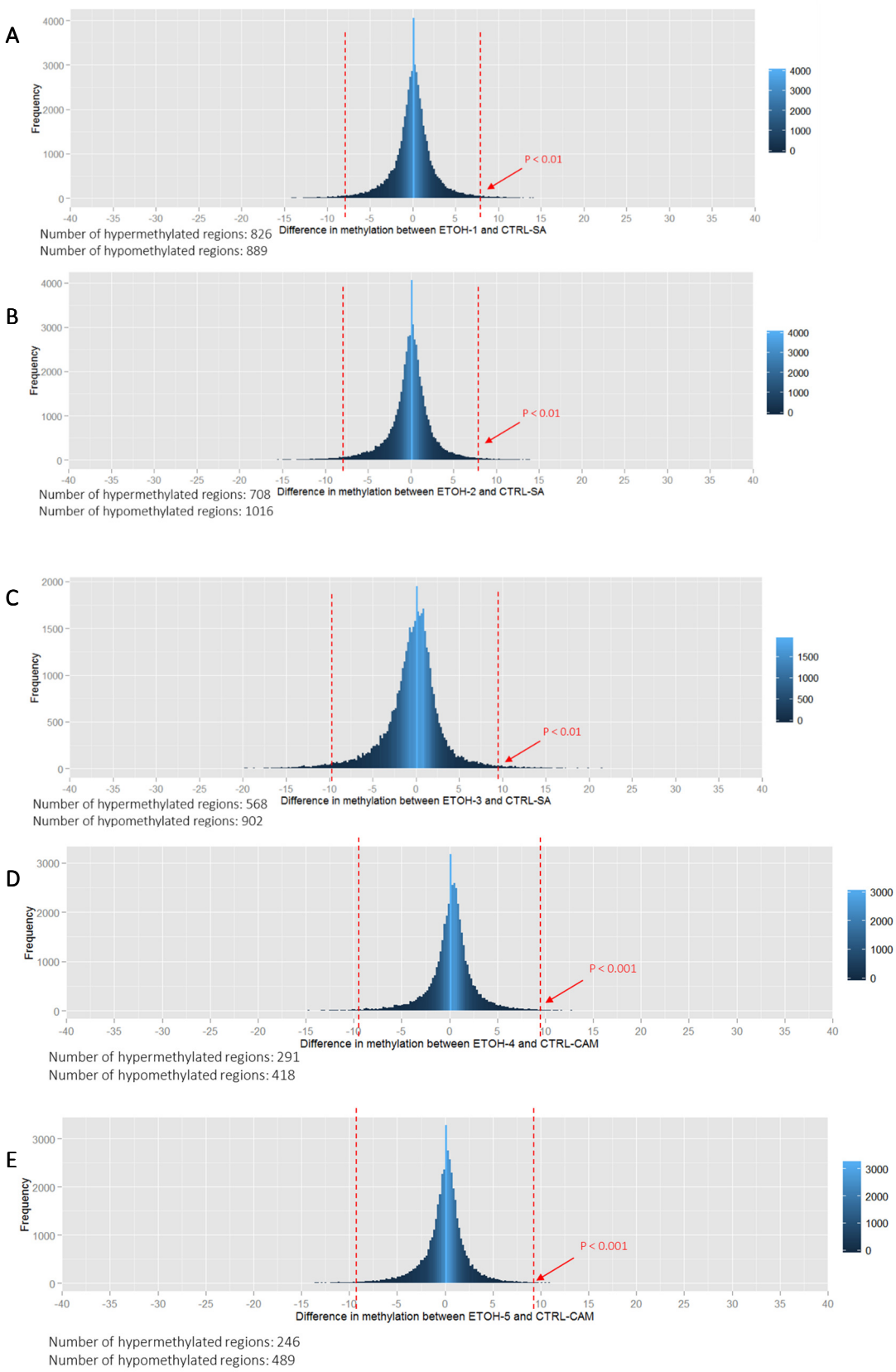
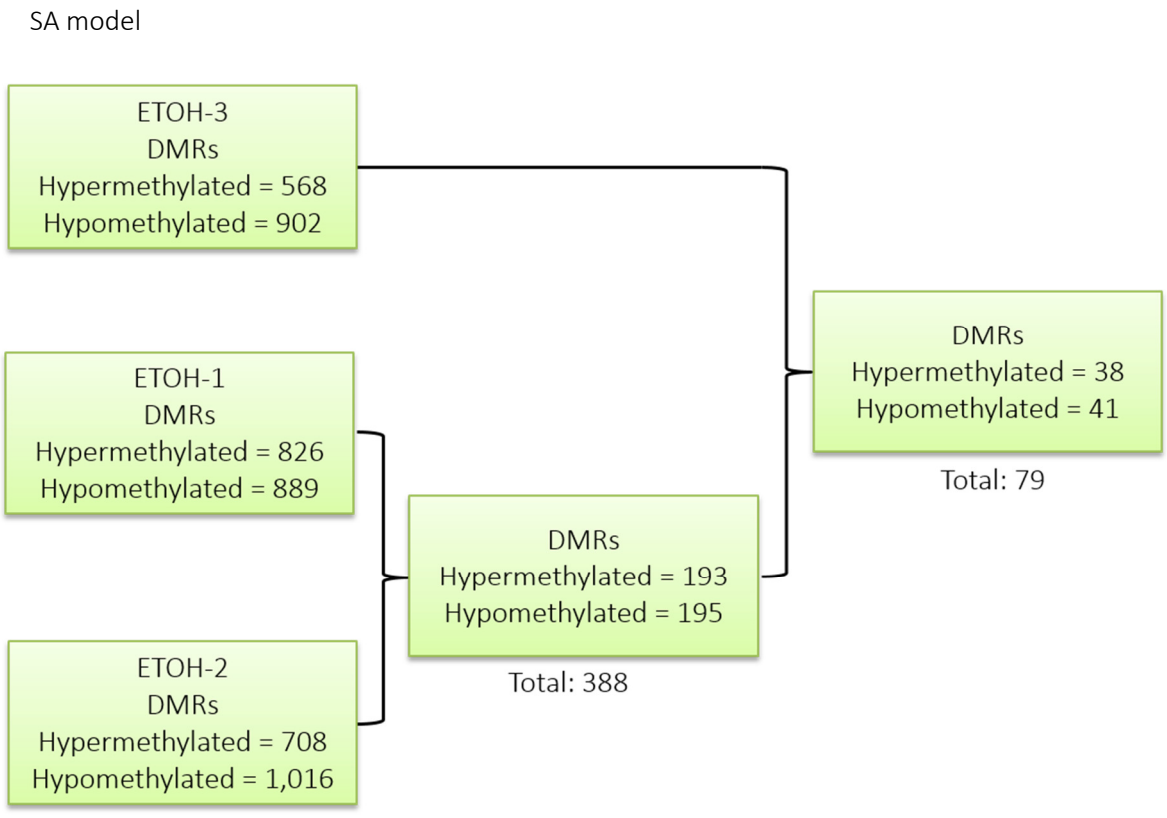


Figure 27: Distribution of differentially methylated regions (DMRs). The binomial distribution of the degree to which each region from ETOH pools differed to that of corresponding CTRL pool was plotted. The first three figures depict that of the SA model (where a cut-off of $p<0.01$ was used). The second two figures depict that of the CAM model (where a cut-off of $p<0.001$ was used).

A total of 1,715; 1,724; 1,470; 709 and 735 significantly differentially methylated regions were identified in pools ETOH-1, -2, -3, -4 and -5, respectively (Figure 28). DMRs which demonstrated reduced levels of DNA methylation when compared to the control pool (hypomethylated DMRs: 889; 1,016; 902; 291; 246 in ETOH-1, -2, -3, -4 and -5, respectively) and those which demonstrated increased DNA methylation in comparison to the control pool (hypermethylated DMRs: 826, 708, 568, 418, 489 in ETOH-1, -2, -3, -5 and -5, respectively) were evident in all ethanol pools.



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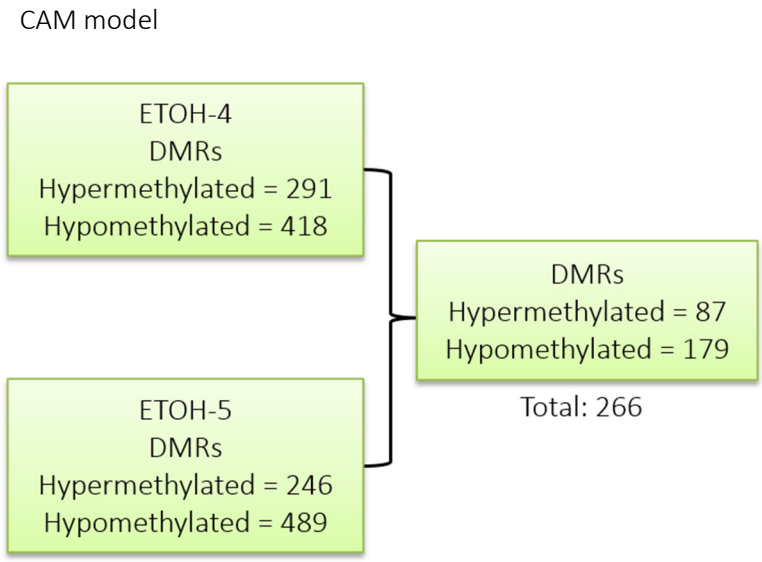


Figure 28: Overview of commonly identified significantly differentially methylated regions. The top figure depicts the common DMRs identified in the SA model (p<0.01), while the bottom figure depicts the common DMRs identified in the CAM model (p<0.001).

In order to identify significantly altered DMRs (significant DMRs) common to all ETOH pools, a pair-wise comparison was made between overlapping DMRs in each pool (Figure 28). Due to the fact that ETOH-3 was the data-limiting pool in the SA model, pools EtOH-1 and EtOH-2 were compared first for common significant DMRs. A total of 388 common significant DMRs were identified (n=193 hypermethylated, n=195 hypomethylated) (). This common set of significant DMRs was then compared with pool ETOH-3. This comparison yielded 79 common significant DMRs (n=38 hypermethylated, n=71 hypomethylated). Similarly, the CAM model (ETOH-4 compared to ETOH-5) yielded 266 common significant DMRs (n=87 hypermethylated, n=179 hypomethylated) (APPDENDIX).

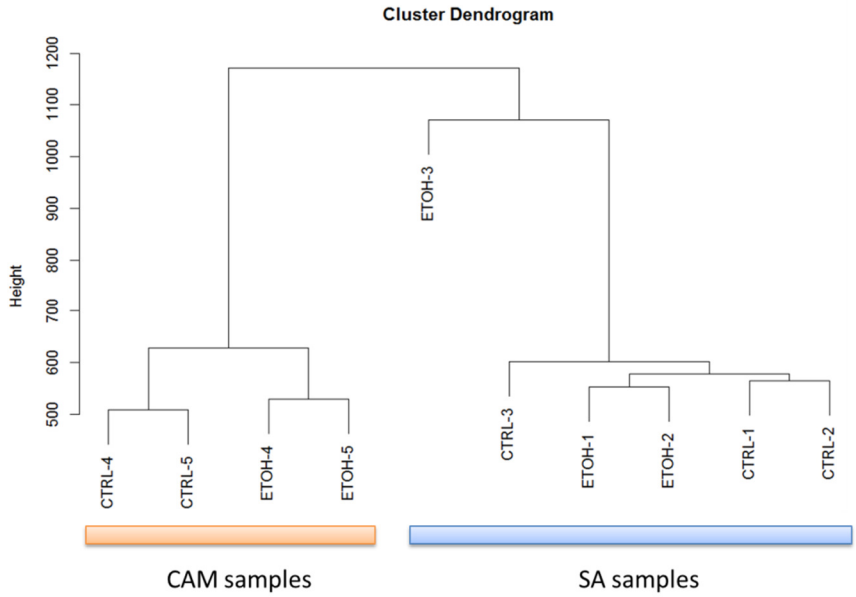
Comparison of RRBS DNA methylation data between the SA and CAM models

In order to determine DMRs common to both the SA and CAM models, a total of 13,274,487 500bp windows with a stride of 200bp were assessed across all RRBS pools. Each ETOH pool was compared to its corresponding CTRL pool for each model (Table 7). Post clustering analysis was performed, which represents the number of windows that encompass ≥1 informative CpG (with a read depth of ≥5) after clustering adjacent and overlapping 500bp windows. This yielded approximately 84,000 – 105,000 regions (background regions) across all pools. Pools were then filtered for windows with ≥3 informative CpGs, which yielded approximately 44,000 – 56,000 background regions across all pools. The data-limiting pool ETOH-3 was omitted from further analyses. A total of 52,881 background regions were identified in the SA model (regions common to both ETOH-1 and ETOH-2), and 43,922 background regions were identified in the CAM model (background regions common to ETOH-4 and ETOH-5) with an overlap fraction of >0.8 (>80%). As can be observed there was similarity in RRBS coverage between the two modesl. This suggests that although RRBS only covers a very small percentage of the genome (typically ~5% of all CpGs) RRBS tends to capture representative selections of genomic regions, such as CpG islands, shores, and a substantial number of low-methylation regions (LMRs).

Table 7: Identification of common regions in both SA and CAM model RRBS data.

Model	Comparison	Post Clustering (≥1 CpG dinucleotide)	Window (≥3 CpG dinucleotides)	Background regions (overlap with fraction > 0.8)
SA	ETOH-1 vs CTRL-SA	105,255	56,230	52,881
	ETOH-2 vs CTRL-SA	105,687	56,428	
	[ETOH-3 vs CTRL-SA	86,812	44,436]	-
CAM	ETOH-4 vs CTRL-CAM	84,633	44,761	43,922
	ETOH-5 vs CTRL-CAM	90,631	47,995	

Principal components analysis (PCA) and hierarchical clustering analyses were performed using the overlapped common regions (with fraction > 0.8) between SA model captured regions (n=52,881) and CAM model captured regions (n=43,922) (Figure 29). It was found that there were 38,881 common regions. Further to this, we observed that there appeared to be global differences in DNA methylation between the SA model and the CAM model. Within each cluster (blue and yellow clusters in Figure 29) we again observe that ETOH and CTRL samples cluster separately. This is also evident in the dendogram cluster. As expected, we observed the distant clustering of the data-limiting "problem" sample, pool ETOH-3, which clusters separately from the SA model cluster.



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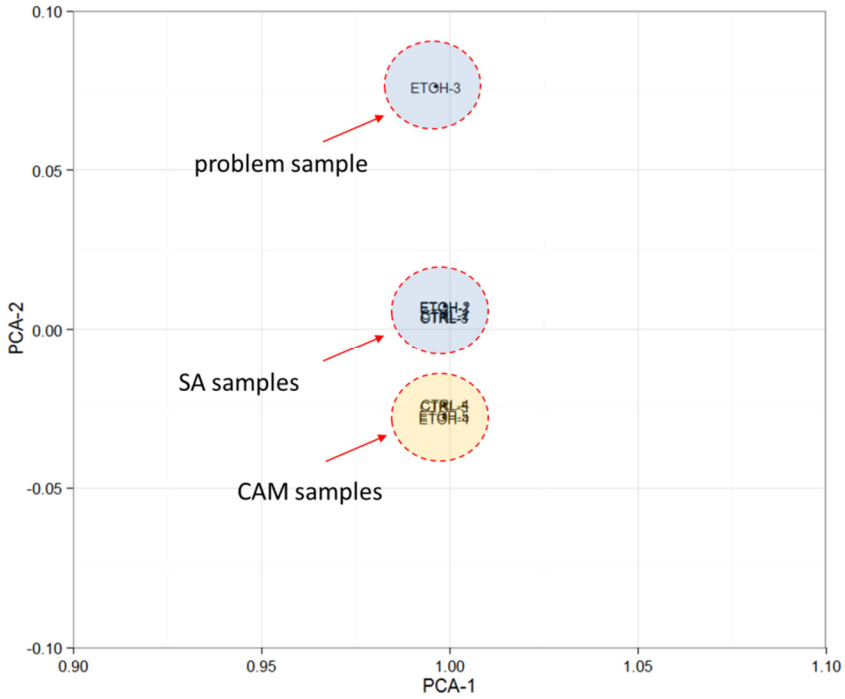

Figure 29: Cluster analysis of SA and CAM model RRBS DNA methylation data. Top figure depicts a dendogram cluster of the SA and CAM model RRBS data. Bottom figure depicts a PCA plot of the SA and CAM models. In both figures, it is observed that: the two models cluster separately; within each cluster, ETOH and CTRL data cluster separately; and the data-limiting "problem" sample, pool ETOH-3, clusters separately from the SA model cluster.

We next investigated the DMRs that were common to both the SA and CAM models. Our analysis revealed that eight DMRs were common to both models (Table 8), which were observed with a probability that is significantly higher than if we picked them by chance (see bootstrapping analysis in Figure 30). These DMRs included *Atp6v1h*, *Zfp704*, *Dnahc10*, *Tgfa*, *Vwf*, *Tet1*, *Sema4g*, and *Sorcs3*.

Table 8: Significantly differentially methylated regions in both the SA and CAM models from sperm RRBS analysis.

Gene	Description
Atp6v1h	*ATPase, H+ transporting, lysosomal V1 subunit H*
Zfp704	*zinc finger protein 704 Mus musculus*
Dnahc10	*dynein, axonemal, heavy chain 10*
Tgfa	*transforming growth factor alpha*
Vwf	*Von Willebrand factor homolog*
Tet1	*Ten-Eleven Translocation 1*
Sema4g	*sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G*
Sorcs3	*sortilin-related VPS10 domain containing receptor 3*

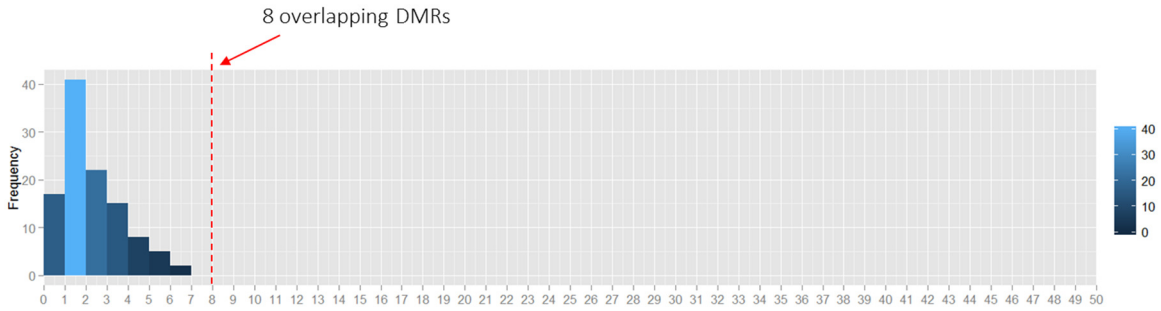

Figure 30: Bootstrap analysis of the probability of finding eight overlapping DMRs by chance. A bootstrap analysis was performed using 100 iterations. Mean = 1.81, median = 1.45, standard deviation = 1.45. Red dashed line and arrow indicate that the eight overlapping DMRs were observed with a probability (p<0.01) that is greater than chance (indicated in the frequency distribution in blue).

Identification of low-methylated regions (LMRs) in the SA model

Low-methylated regions (LMRs) are genomic regions which demonstrate DNA methylation of between 20% and 40%. Of the 79 common significant DMRs identified in the SA model, 17 were found to be LMRs (21.5%; χ^2 $p<0.0001$). Of the 388 DMRs common to pools EtOH-1 and EtOH-2, 90 were LMRs (23.2%; χ^2 $p<0.0001$). This is in contrast to the 4.6% (2,424 of 52,881) LMRs observed in the non-significant background regions.

2.3.1. Assessment of hemi-methylation across the sperm epigenome

Chronic alcohol exposure has been shown to decrease Dnmt mRNA levels in sperm of rats (Bielawski et al., 2002); inhibit foetal DNA methylation via decreased DNA methylase activity (Garro et al., 1991); and prevent normal methylation programming of key neural stem cell genes during NSC differentiation (Zhou et al., 2011a). It has also been shown that chronic exposure of alcohol decreases DNMT3b mRNA levels in the sperm of male rats, which is associated with DNA hypermethylation (Bonsch et al., 2006).

It is therefore possible that chronic alcohol exposure is able to inhibit the maintenance DNA methyltransferase protein, Dnmt1. The consequence of this inhibition would impact on the maintenance or faithful replication, of DNA methylation marks from a parental DNA strand to its nascent daughter strand during cellular division. It is therefore plausible that the continual inhibition of Dnmt1 activity during gametogenesis (spermatogenesis) as a result of chronic exposure of male mice to ethanol would increase the occurrence of hemi-methylated DNA in sperm DNA.

To investigate whether there was evidence of enrichment for hemi-methylated DNA, the SA and CAM sperm DNA datasets were filtered, so that only CpG dinucleotides with both CpG

cytosines (on both strands) mapped with ≥5 reads were included (summarised in Table 9 below).

Table 9: Summary of hemi-methylation analysis of sperm DNA from treated male mice.

Sample origin	Sample ID	Male Treatment	No. CpGs with partner ≥5 reads	No. CpGs with both CpGs informative (≥5 reads)	No. hemi-methylated CpGs	% hemi-methylation
SA model	EtOH-1	EtOH	231,554	115,777	539	0.47%
	EtOH-2	EtOH	235,172	117,586	600	0.51%
	EtOH-3	EtOH	105,820	52,910	266	0.50%
	CTRL-1	Sucrose	230,848	115,424	596	0.52%
	CTRL-2	Sucrose	236,894	118,447	580	0.49%
	CTRL-3	Sucrose	223,524	111,762	559	0.50%
CAM model	EtOH-4	EtOH	248,840	124,420	850	0.68%
	EtOH-5	EtOH	236,260	118,130	630	0.53%
	CTRL-4	Sucrose	224,588	114,294	578	0.51%
	CTRL-5	Sucrose	211,166	105,583	502	0.48%

Approximately 220,000 CpG cytosines were observed to have a partner cytosine on the same CpG site. Hemi-methylation was defined as a CpG site where the cytosines on either strand at that position showed a ≥60% difference in DNA methylation.

The percentage of hemi-methylation for each RRBS datasets/sperm DNA pool was calculated (Table 9). Each pool demonstrated approximately 0.5%. Although pool ETOH-4 showed a comparably higher level of hemi-methylation than other samples, pool ETOH-5 from the same biological background (Ethanol treated, CAM pool ETOH-5) did not show the same difference. Overall hemi-methylation levels were not significantly different between the two

groups (Mann-Whitney *p*=0.548), although considerable variation in hemi-methylation was observed in the ethanol treatment group (Figure 31).

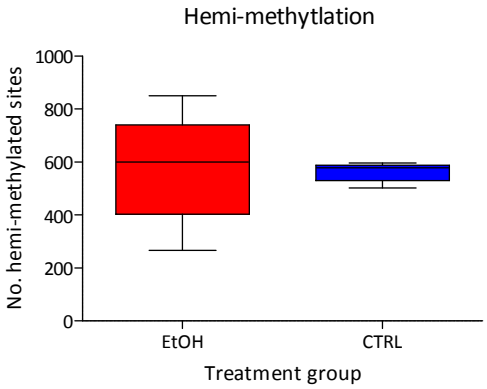

Figure 31: Comparison of hemi-methylation between the ETOH and CTRL male groups.

Gene ontology analysis of significant DMRs

Genes within the closest proximity to the 79 significant DMRs common to all three ETOH pools of the SA model were used to perform a gene ontology analysis using DAVID (PMID: 19131956 and 19033363). DAVID is one of the most commonly used tools for functional enrichment analyses of gene lists. DAVID provides an integrated approach to identify functions enriched in the gene list and clusters them according to their similarity. Functions include gene ontology, protein sequence annotation (using Swiss-Prot and InterPro matches), known disease associations with a genetic components (using the Online Mendelian Inheritance in Man (OMIM) database), and associations of genes with biological pathways (using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database). The gene enrichment is supported by a modified Fisher's Exact test and multiple testing corrections to

ensure that the functional enrichment observed is not due to chance. The top six functional annotation clusters are shown in Figure 32 and Figure 33. The "high" stringency parameter was used.

Functional Annotation Clustering – 79 DMRs

Annotation Cluster 1	**Enrichment Score: 1.4**			**Count**	**P_Value**	
SP_PIR_KEYWORDS	immune response	RT		4	1.3E-2	*Inppl1, Polr3g, Mif, Tlr3*
SP_PIR_KEYWORDS	innate immunity	RT		3	1.7E-2	
GOTERM_BP_FAT	innate immune response	RT		3	3.4E-2	
GOTERM_BP_FAT	defense response	RT		3	3.5E-1	
Annotation Cluster 2	**Enrichment Score: 1.09**			**Count**	**P_Value**	
GOTERM_BP_FAT	sexual reproduction	RT		5	2.0E-2	*Crtap, Cxcl12, Mtap7, Ovgp1, Spata5*
GOTERM_BP_FAT	gamete generation	RT		4	6.1E-2	
GOTERM_BP_FAT	reproductive process in a multicellular organism	RT		4	9.9E-2	
GOTERM_BP_FAT	multicellular organism reproduction	RT		4	9.9E-2	
GOTERM_BP_FAT	male gamete generation	RT		3	1.5E-1	
GOTERM_BP_FAT	spermatogenesis	RT		3	1.5E-1	
Annotation Cluster 3	**Enrichment Score: 0.79**			**Count**	**P_Value**	
GOTERM_MF_FAT	cation binding	RT		14	1.1E-1	*Emr1, Prdm6, Creb5, Yaf2, Adcy6, Alox12e, Celsr3, Cpe, Lctl, Ovgp1, Tktl2, Zfp64, Zfp704, Zdhhc23*
GOTERM_MF_FAT	ion binding	RT		14	1.2E-1	
GOTERM_MF_FAT	metal ion binding	RT		12	3.1E-1	
Annotation Cluster 4	**Enrichment Score: 0.76**			**Count**	**P_Value**	
SP_PIR_KEYWORDS	extracellular matrix	RT		3	1.1E-1	*Emid2, Vwf, Crtap*
GOTERM_CC_FAT	proteinaceous extracellular matrix	RT		3	2.1E-1	
GOTERM_CC_FAT	extracellular matrix	RT		3	2.2E-1	
Annotation Cluster 5	**Enrichment Score: 0.57**			**Count**	**P_Value**	
INTERPRO	Zinc finger, C2H2-like	RT		4	2.4E-1	*Prdm6, Creb5, Zfp64, Zfp704*
INTERPRO	Zinc finger, C2H2-type	RT		4	2.5E-1	
SMART	ZnF_C2H2	RT		4	3.2E-1	
Annotation Cluster 6	**Enrichment Score: 0.56**			**Count**	**P_Value**	
UP_SEQ_FEATURE	transmembrane region	RT		17	1.1E-1	*Htr2c, Abcc6, Abcg3, Emr1, Gprc5b, Adcy6, Celsr3, Ccdc109a, Edar, Lctl, Lmf1, Olfr523, Prtg, Slco1b2, Sorc3, Tlr3, Unc80, Zdhhc23*
SP_PIR_KEYWORDS	transmembrane	RT		18	1.9E-1	
GOTERM_CC_FAT	integral to membrane	RT		18	4.9E-1	
GOTERM_CC_FAT	intrinsic to membrane	RT		18	5.7E-1	

Figure 32: DAVID gene ontology analysis of the 79 significant common DMRs.

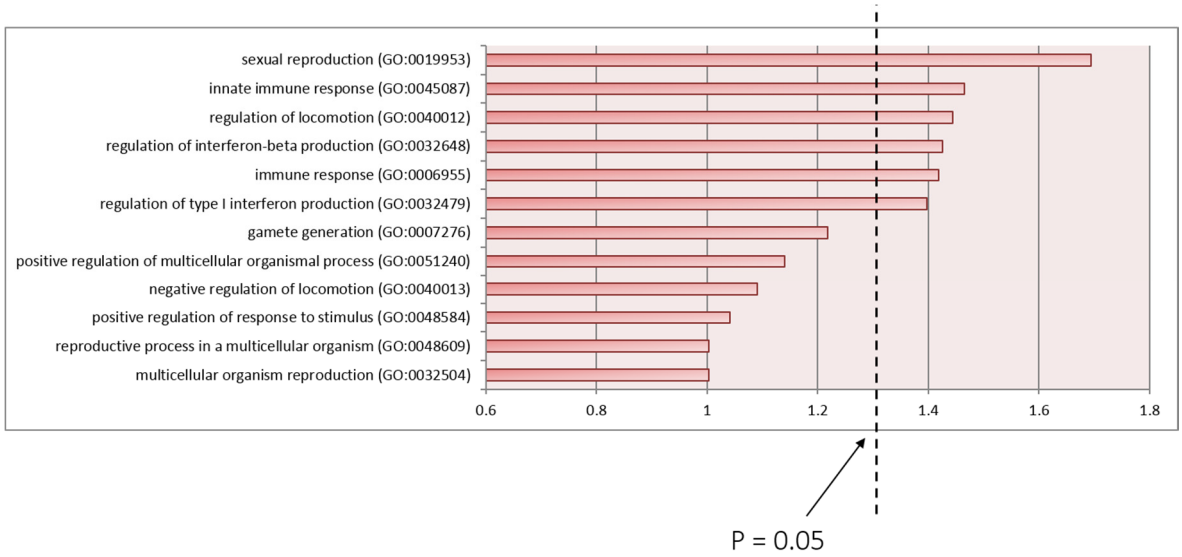

Figure 33: DAVID Gene Ontology of the SA model RRBS sperm DMRs. The DAVID Gene Ontology analysis of the 79 common significant DMRs revealed six significantly enriched biological processes (p=0.05): sexual reproduction, innate immune response, regulation of locomotion, regulation of interferon-beta production, immune response, and regulation of type I interferon production.

A similar gene ontology analysis was performed for the two ETOH pools that comprised the CAM model using the 266 common significant DMRs. Biological processes including glycosylation, regulation of cell cycle, cytoskeleton and neural development appeared to be affected, and thus enriched (Figure 34).

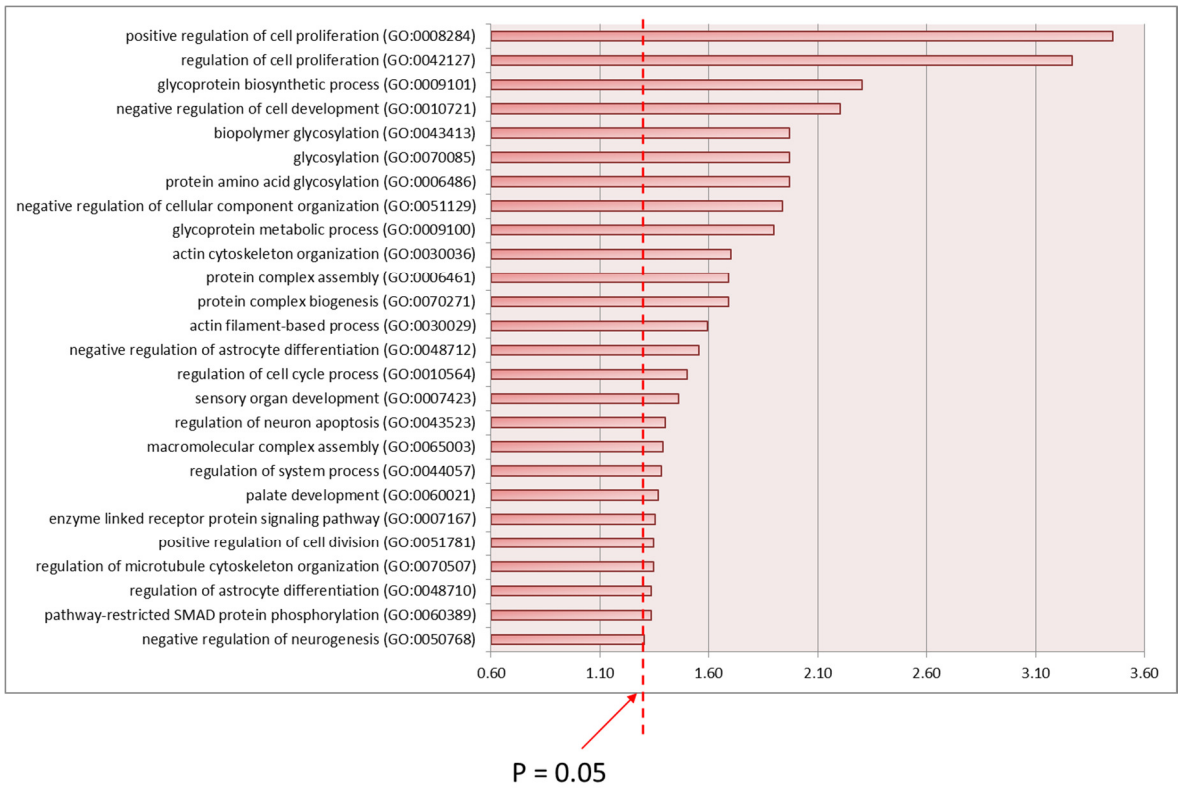

Figure 34: DAVID Gene ontology analysis of CAM model RRBS sperm DMRs. GO analysis of the 266 DMRs identified in the CAM model revealed 26 significantly ($p \leq 0.05$) enriched biological processes. These encompassed biological processes that included glycosylation, regulation of cell cycle, cytoskeleton, and neural development.

Functional annotation overlap between significant DMRs from the SA and CAM models

In order to investigate whether similar biological processes were enriched for in the gene ontology analyses from the two models (SA and CAM), a functional annotation clustering overlap analysis was performed, where genetic sequences (typically protein coding regions) are functionally characterised and classified, and are then clustered into common functions. This overlap analysis made use of the 388 significant common DMRs identified from the ETOH-1/ETOH-2 comparison (SA model), and the 266 significant common DMRs identified from the ETOH-4/ETOH-5 comparison (CAM model). This analysis identified main themes (biological processes) that were common to both models, along with the genes that comprised each biological process (Table 10). Biological processes that were common to

both models included neuron development, immunity, glycoprotein metabolism, cytoskeleton, cell motility/mobility, blood vessel development, heart development, sexual reproduction spermatogenesis, and gamete generation.

Table 10: RRBS Gene ontology biological processes overlap between SA and CAM models

SA model	CAM model	SA model	CAM model
Neuron development and differentiation	Neuron development, neurogenesis	Blood vessel development	Blood vessel development
Rtn4rl1	*Nf1*	*Fgf6*	*Nf1*
Pip5k1c	*Id4*	*Flt1*	*Tgfbr3*
Celsr3	*Ephb2*	*Tgm2*	*Tgfa*
Cxcl12		*Tgfa*	*Arhgap24*
Ephb1	Sensory organ development	*Angpt2*	*Tgfb2*
Nkx6-1	*Kcnma1*	*Eng*	
Alcam	*Tshz1*	*Cxcl12*	
Lif	*Nf1*		
Gata2	*Pax2*	Heart development	Heart development
Slitrk3	*Ephb2*	*Tsc1*	*Nf1*
Spnb4	*Dscam*	*Tbx5*	*Tgfbr3*
Abt1	*Tgfb2*	*Rpgrip1l*	*Zfpm1*
Sema4f		*Tead1*	*Tgfb2*
Nrg1		*Sox9*	
		Nrg1	
Immune response	Immune system development	*Eng*	
Lif	*Tgfbr3*		
Polr3g	*Cbfa2t3*	Sexual reproduction	Sexual reproduction
Ccl25	*Tgfb2*	*Ovgp1*	*Gm14374*
Adora3	*Il31ra*	*Spnb4*	*Lmna*
Procr		*Stx2*	*CTCFL*
Inppl1		*Crtap*	*Dazl*
Pomp		*Ggt1*	*Bcl2l1*
Tlr3		*Mtap7*	*Diap2*
Malt1		*Spata5*	*Ggnbp1*
Il1rapl1		*Cxcl12*	
Cxcl12			
Mif		Spermatogenesis	Spermatogenesis
		Crtap	*Gm14374*
Glycoprotein metabolism	Glycoprotein metabolism	*Ggt1*	*Lmna*
Stt3b	*St3gal1*	*Mtap7*	*Dazl*
Fut8	*Slc4a10*	*Spata5*	*Bcl2l1*
B3galnt2	*St6galnac3*		*GGNBP1*
Man1c1	*B3gnt4*		
	Csgalnact2	Gamete generation	Gamete generation
	Mgat5		
		Regulation of cell proliferation	Regulation of cell proliferation
Cytoskeleton	Cytoskeleton	*Malt1*	*Odc1*
Shroom1	*Cgnl1*	*Mtap7*	*Fgfr4*
Ncs1	*Arhgef2*	*Sox9*	*Flt4*
Dnahc10	*Nos1*	*Cxcl12*	*Nf1*
Pdlim1	*Shroom3*		*Cntfr*
Ptpn21	*Ttll9*		*Bcl2l1*
Pxn	*Lmna*		*Tgfb2*
Plcb4	*Ncs1*		*Il31ra*
Spnb4	*Krt12*		*Cd38*
Procr	*Dnahc10*		*Grpr*
Mtap7	*Arhgap24*		*Tgfa*

Wipf1	*Was*		*Tgfbr3*
Mtus2	*Mtap1b*		*Id4*
Obscn	*Plec*		*Asph*
Sh3pxd2a			*Etv4*
Inppl1			
Rpgrip1l		Regulation of cell communication	
Ptpn13		*Lif*	
Ky		*Adora3*	
Sfi1		*Klb*	
Csrp1		*Kl*	
Dnmbp		*Tgm2*	
Cass4		*Ncs1*	
Dnahc5		*Tgfa*	
Klhl17		*Tlr3*	
Lca5		*Nrg1*	
Myo15			
Clip2		Placenta development	
Myh14		*Lif*	
Dynlrb2		*Vwf*	
		GATA2	
Cell mobility	Cell motion	*Arnt*	
Ccl25	*Etv4*		
Flt1	*Ephb2*	Wound healing	
Dnahc5	*Tgfb2*	*Vwf*	
Fut8		*Lnp*	
Nrg1		*Procr*	
Selplg		*Entpd1*	
Cxcl12			Cellular homeostasis
			KCNMA1
			GJD2
			TRPC6
			NF1

2.4. Discussion

Studies investigating the effect of prenatal alcohol exposure on DNA methylation, such as that by Kaminen-Ahola et al. (2010), commonly focus on specific genes. Kaminen-Ahola made us of the epigenetically-sensitive *Agouti viable yellow* (A^{vy}) murine locus. A^{vy} is commonly used as an epigenetic biosensor for environmental effects on the foetus, because prenatal exposure of both nutritional and toxic genes has the ability to effect A^{vy} expression in the offspring through epigenetic regulation. For instance, in cases of hypomethylation of the A^{vy} promoter element there is constitutive expression of the *Agouti* gene and is reflected by a yellow coat colour. To the contrary, hypermethylation is correlated with promoter

silencing and the generation of a brown pseudo-agouti coat colour, while a mottled coat colour is the result of intermediate expression of A^{vy}. Kaminen-Ahola et al. (2010) examined the effect of gestational ethanol exposure in A^{vy} heterozygous mice and found that the proportion of pseudo-agouti offspring increased as a result of ethanol exposure. This was linked to higher levels of transcriptional silencing which is consistent with a decreased expression of A^{vy} due to hypermethylation. This study highlighted the ability of prenatal alcohol exposure to alter the foetal epigenotype and, consequently the adult phenotype. In addition, Kaminen-Ahola et al. (2010) observed growth restriction, certain craniofacial dysmorphologies and altered gene expression profiles in the livers of their ethanol-exposed wild type (a/a) siblings, phenotypes that are all associated with FASD.

Similarly, following in utero ethanol exposure Downing et al. (2011) examined the DNA methylation and gene expression patterns in embryonic tissue at the mouse *Igf2* locus. They observed a reduction in methylation in the embryo at *Igf2*, the locus responsible for encoding insulin growth factor 2. The reduction in methylation was associated with a decrease in expression of *Igf2*. Additionally, the group observed malformations of the skeletal system comparable to those observed in FASD patients, which include vertebral and rib malformations, and short metacarpal bones (CDC, 2004). Taken together, these findings imply that DNA methylation may play an important role in the mechanisms underlying FASD. Together, these findings provide compelling evidence for alcohol induced epigenetic alterations.

In 2009 Ouko et al. (2009) aimed to investigate whether a link existed between human male alcohol use and hypomethylation of imprinted loci within their sperm DNA, regions considered to be important for embryonic development. This was undertaken to investigate a potential mechanism for paternal effects in the aetiology of FASD. They observed hypomethylation (lower-than-normal methylation) within the *IG*-DMR in sperm DNA of

males who consumed chronic amounts of alcohol, suggesting that these alterations may be passed on from the males through fertilisation which may lead to the development of a FASD-like phenotype in their offspring. With a similar attempt, our Knezovich and Ramsay (2012) investigated the DNA methylation patterns at two paternally methylated imprinting control regions (*H19* and *Rasgrf1*) in the sperm of exposed male mice and somatic DNA of their sired offspring following preconception paternal alcohol intake. We observed significant reductions at the *H19* CCCTC-binding factor 1 (CTCF 1) (p= 0.0027) and CTCF 2 (p= 0.0009) binding sites in the offspring of ethanol-treated males, which was associated with a reduction in weight at postnatal days 35-42 (p< 0.05).

Our present study examined the sperm methylome of male mice chronically exposed to ethanol, using RRBS, which enriches for CpG islands across the genome. Methylation levels at captured CpG islands were compared to those of controls to identify regions of significant differentially methylation.

2.4.1. Chronic alcohol exposure alters sperm DNA methylation across the genome

Conventional CpG islands contain an observed-to-expected CpG ratio of 60% or greater (Takai and Jones, 2002). However, paternal ICRs contain poorly defined CpG islands, in that they generally contain an observed-to-expected CpG ratio of approximately 40% (Schulz, 2010). This is in contrast to high and intermediate CpG-content promoters (which contain traditional CpG islands) which have an observed-to-expected CpG ratio of approximately 50% or greater. RRBS optimally captures CpG islands with a minimum observed-to-expected ratio of 60% and a GC content of 0.5 (Gu et al., 2011). This constitutes approximately 27,458 and 44,440 bona fide CpG islands across the mouse and human genomes, respectively, which include approximately 20,746 and 23,690 gene promoters within the mouse and

human genome, respectively (representative of approximately 1% of both genomes) (Gu et al., 2011). Therefore, because paternal ICRs contain an observed-to-expected CpG ratio that is lower than that optimally captured by RRBS, paternal ICRs were not captured by the current study's RRBS. Thus, locus specific pyrosequencing was employed to quantify DNA methylation at paternal ICRs.

2.4.1.1. Alcohol appears to have nuanced effects on paternal ICRs

Quantitative pyrosequencing performed in our study to investigate the effect of chronic alcohol exposure on paternally imprinted ICRs revealed no significant changes in DNA methylation at the *IG*-DMR, *H19* ICR and *Rasgrf1* regions in sperm DNA. A significant change in DNA methylation was only observed at a single site, where a 1.33% reduction was observed at the *IG*-DMR CpG2 sites in the sperm of alcohol treated males (p=0.03) (Table 5).

Contrary to our findings, the effect of direct environmental exposures on differentially methylated regions (DMRs, in terms of imprinted loci) and imprinting control regions (ICRs) have been observed in several studies. As summarised in Parle-McDermott and Ozaki (2011), the nutritional constituents of culture media, the in utero environment, and adult diet can have a significant effect on DNA methylation sites across the genome (summarised in Table 11). Regions affected include environmentally sensitive genes (*Agouti* and *Axin*^{Fu}), non-imprinted regions enriched with CpG sites (CpG islands, promoter regions) and imprinted regions (*Igf2*, *H19* and *MEG3* – all of which contain ICRs).

Table 11: Summary of nutrition-sensitive methylation sites in mice and humans.

Nutrient	Model	DNA methylation site(s) examined
Methyl donor supplementation	Maternal mouse offspring	Agouti gene
		Cdk5 activator binding protein
		Axin^{Fu} gene
		82 genes including runx3
Methyl donor deficiency	Maternal sheep offspring	1400 CpG islands
	Adult rats	*p53*
	Postweaning mouse	*Igf2*
	Pregnant mice	*Esr1, Igf2, Slc39a4CC*
	Rat brain	Global methylation
Choline deficiency	Maternal mouse offspring	*Cdkn3*, global methylation
	Maternal rat offspring	*Dnmt1, Igf2*, global methylation
Protein restriction	Adult rats, F2 offspring	*PPARα*
	Maternal mouse offspring	CpG islands including *Lxrα*
	Cultured mouse blastocysts	*H19*
	Paternal rat offspring	*PPARα*: CpG island 50kb upstream
	Adult mice	*Leptin* promoter
	Maternal rat offspring/leptin injections	*PPARα*, glucocorticoid receptor
High-fat diet	Adult rats	*Leptin* promoter
Genistein	Adult mice	Differential method including ribosomal DNA and desmin-binding fragment
Genistein supplementation	Maternal mouse offspring	*Agouti* gene
Calorie restriction	Maternal offspring	*IGF2, INSIGF* in adults
		IL10, LEP, ABCA1,GNASAS, MEG3
Folate status	Adults/MTHFR 677C > T genotype	Global methylation
Folate, vitamin B-12 status	Adults: colon	*ERα, MLH1*
Folic acid consumption	Maternal offspring	*IGF2*
Folic acid intervention	Postmenopausal women	Global methylation
Folate/homocysteine status	Cord blood	14,496 genes
Soy isoflavone intervention	Premenopausal women	*RARβ2, CCND2, p16,RASSF1A, ER*

Table obtained from(Parle-McDermott and Ozaki, 2011)

Investigations into the effect of alcohol on DNA methylation contained within paternal ICRs of male gametes have suggested that direct exposure of sperm to alcohol can elicit changes in DNA methylation at ICRs. Ouko et al (2009) observed decreased DNA methylation at sites contained within the *IG*-DMR (CpG 4) and *H19* ICR (CpG 7) in the sperm obtained from adults males who were classified as heavy drinkers. Using a mouse model, Stouder et al (2011) demonstrated that direct exposure of the embryo to alcohol (0.5g/kg) significantly decreased DNA methylation at the *H19* ICR in offspring sperm. Furthermore, they demonstrated that this alcohol-induced hypomethylated state was transgenerationally inherited, having noted reduced *H19* ICR DNA methylation levels in F2 offspring brains. However, although Stouder et al (2011) reported a statistical significance (p<0.005) in the decrease in DNA methylation observed in the offspring of alcohol-exposed dams, this was based on the average methylation of eight CpG sites contained within the CTCF2 binding region of the *H19* ICR, with a methylation change of 3%. Our previous research (Knezovich and Ramsay, 2012) investigated the effect of chronic (3g/kg/day) alcohol exposure on paternal ICR DNA methylation in the sperm of male mice. It was observed that significant reductions at only CpG site 7 in the *H19* ICR CTCF2 binding site in the sperm of alcohol exposed males. More recently, Liang et al (2014) observed that preconception alcohol exposure (3.3 g/kg) of male mice affected DNA methylation at *H19* and *Peg3* (both imprinted genes) in the sperm of exposed mice, and that of *Peg3* (CpG 7 and CpG 11) and *Snrpn* in the cerebral cortices of sired offspring. Findings of this latter study are similar to that of our current study, were site-specific nuanced effects of alcohol exposure on sperm DNA methylation at paternal ICRs was observed. Furthermore, using a mouse model to investigate the effects of prenatal under-nutrition on DNA methylation at imprinted genes, Radford et al (2012) observed that maternally imprinted genes *Peg3* and *Snrpn* were aberrantly hypomethylated in F1 offspring as a consequence of in utero undernourishment.

However, they did not observe changes in DNA methylation at the paternally imprinted ICRs *IG-DMR* and *H19*.

In summary, the data presented in this study is supported by several other studies, and demonstrates that the effect of environmental exposures, particularly that of alcohol, on paternal ICRs is nuanced, or at least, limited to only a subset of CpG sites contained within ICRs. This may suggest that paternal ICRs are resilient to the effects of alcohol exposure.

2.4.1.2. Low-methylation regions are sensitive to alcohol exposure

Although the effect of excessive paternal alcohol exposure on the methylation at hypermethylated paternal ICRs appears to be nuanced, our current study observed a significant enrichment of low-methylation regions (LMRs) in common differentially methylated regions (regions that demonstrated a significant difference in DNA methylation between the control and ethanol treated mice) in the ethanol exposed group of males. We observed that 90 of the 388 significant DMRs (23.2%) ($p<0.0001$) common to ETOH-1 and ETOH-2; and 17 of the 79 significant DMRs (21.5%) ($p<0.0001$) common to all three SA model pools (ETOH-1, ETOH-2 and ETOH-3) were LMRs. This is in contrast to our observations of the non-significant background regions, where we observed only 4.6% enrichment for LMRs (2,424 regions of 52,881). This finding suggests that the effects of ethanol is most pronounced in DNA regions with relatively low levels of methylation (10 – 50% (Burger et al., 2013; Stadler et al., 2011).

Several studies have demonstrated that alcohol exposure is able to significantly affect DNA methylation. Laufer et al (2013) demonstrated long-lasting DNA methylation alterations associated with in utero alcohol exposure across the genome, and particularly at imprinted

genes (not ICRs) in embryonic brains. Bielawski et al (2002) showed that paternal alcohol exposure (6g/kg) decreased cytosine methyltransferase mRNA levels in sperm, however they did not investigate whether there were any associated changes in DNA methylation. Although Govorko et al (2012) did not investigate the effect of alcohol exposure on sperm DNA methylation of P1 male rats exposed to alcohol, they did observe that their F1 male progeny transgenerationally transmitted an aberrant *Pomc* hypermethylated promoter domain via their sperm. This finding therefore suggests that alcohol exposure of the P1 sires induced a DNA methylation aberration in the paternal gametes, which was transmitted to their offspring. Together, these findings demonstrate that exposure of male mice to alcohol is able to alter DNA methylation in sperm of selected imprinted genes and promoter regions. These regions are commonly associated with CpG islands, which use DNA methylation as a mechanism to regulate gene activity. Our study supports observations of alcohol-induced DNA methylation changes in the sperm epigenome, but extends this to a genome-wide level. Our study observed considerable changes in DNA methylation contained within CpG islands across the sperm genome from mice chronically exposed to alcohol. That is, we observed DNA methylation changes in CpG islands associated with gene bodies, promoter regions and enhancer regions, but did not observe DNA methylation changes in ICRs. This finding might suggest that (hypermethylated paternal) ICRs are more resilient to the effects of alcohol exposure, while CpG islands contained within gene promoters, bodies and enhancers are more sensitive to these effects.

Additionally, low-methylated regions are generally associated with low CpG density (Stadler et al., 2011) and enhancer regions (which are also low CpG density regions). Enhancers are important in development, as are LMRs, and low CpG density regions are more sensitive to environmental exposures, which can alter DNA methylation (reviewed in Chapter 1). This is supported by our current findings, whereby significantly differentially methylated regions (as

a consequence of alcohol exposure) were enriched with LMRs. From this, it is plausible that the LMRs identified and significantly enriched in the RRBS data from ethanol treated mice, are of regulatory importance.

Interestingly, Zhou et al (2011a) showed that alcohol exposure of neural stem cells in culture affected DNA methylation of genes involved in neural cell differentiation, in both directions (induced both hypomethylated and hypermethylated states). Moreover, they observed this effect primarily at genes with "moderate methylation". It is therefore plausible that these genes are LMRs. This effect of alcohol to cause increased (hypermethylation) and decreased (hypomethylated) DNA methylation at genes with "moderate" methylation is supported in our current study, which found enrichment for LMRs in the genes which showed a significant differential methylation when compared to controls.

In conclusion, our data in conjunction with others, suggests that LMRs are particularly sensitive to the presence of alcohol. Furthermore, LMRs behave in a tissue specific manner and are implicated in the regulation of embryonic development. Govorko (2012), Stouder (2011), Manikkam (2012) and Kobayashi (2009) further demonstrate the possibility that aberrant sperm DNA methylation signatures are transmissible to subsequent generations, which are able to elicit a phenotype in sired offspring. These studies open the possibility for epigenetic inheritance of aberrant LMR DNA methylation profiles observed in the sperm of ethanol-exposed sires in our study, and consequently, the inheritance of compromised epigenetic codes required for developmental programming in subsequent offspring.

2.4.2. Differentially methylated regions are enriched for biological processes related to the phenotype observed in FAS

The overall findings of our study suggest that excessive paternal alcohol exposure is able to alter (increase and decrease) DNA methylation signatures of CpG islands across the sperm methylome. These changes predominantly occurred at LMRs, suggestive of the fact that LMRs are more sensitive to the effects of alcohol (while hypermethylated regions, such as the three paternal ICRs, are more resilient to these effects). GO analysis of the SA model sperm DNA methylation data revealed that six significant biological processes were identified among the top 79 common significantly differentially methylated regions. Broadly speaking, the top biological processes were related to immune response, regulation of locomotion and sexual reproduction. A similar GO analysis of the CAM model sperm DNA methylation data revealed 26 significantly enriched biological processes from the 266 common DMRs. Biological processes that were mainly affected included glycosylation, regulation of cell cycle, cytoskeleton, and neural development. An overlap analysis of the 388 DMRs common to ETOH-1 and ETOH-2 from the SA model and the 266 DMRs common to ETOH-4 and ETOH-5 from the CAM model was performed in order to determine whether there were significant DMRs common to both models (ETOH-3 was omitted from this analysis, as the limited data from this pool limited the analysis, and therefore insight into the overlap analysis). The biological process that were enriched in the overlap analysis included: neuron development; immune system development/response; spermatogenesis/gamete generation; glycoprotein metabolism; regulation of cell proliferation; cytoskeleton; cell mobility/motion; blood vessel development; heart development; and sexual reproduction. Foetal alcohol syndrome or alcohol exposure has been associated with aberrations to several of these biological processes.

2.4.2.1. DMRs are associated with genes involved in neural and heart development

This findings of the current study observed both aberrant increases and decreases in DNA methylation across the sperm methylome as a result of alcohol exposure. Moreover, it was observed that a subset significant DMRs in both the CAM and SA alcohol exposure models were associated with neural development (Table 10). As has been observed in *in utero* exposure studies, alcohol has a significant effect on neural development, which may be a consequence of epigenetic dysregulation. Ogawa et al (2005) demonstrated that neural tube formation is highly sensitive to *in utero* alcohol exposure during neurulation (E8-10). These authors further noted that alcohol exposure was associated with significant changes in DNA methylation, which was correlated with the severity of neural tube defects (Liu et al., 2009). Both increased and decreased DNA methylation patterns were observed across the 19,044 promoters investigated in embryos exposed to alcohol. Although this highlights the potential role of epigenetic mechanisms in neural development, where dysregulation of these mechanisms (particularly DNA methylation) due to alcohol exposure is associated neural deficits, caution must be taken as neural and cardiovascular developmental ontologies tend to be overrepresented in enrichment analyses.

In 2011, Zhou et al (2011c) investigated the effect of alcohol on gene expression in embryos exposed to alcohol in culture. They found that 15 gene sets were significantly enriched in their gene expression data. Further GO analysis revealed enrichment of biological processes that included: growth regulation (cell growth), eye and heart (angiogenesis) development, epigenetics (nucleosome modelling), and growth retardation. Furthermore, these enriched biological processes were associated with neural tube defects. Specifically, these authors observed reduced expression of neural specification genes and neurotrophic/growth factor

genes. The GO analyses conducted in our current study, also found enrichment for neuron development, as well as sensory organ and heart development (Table 10).

In addition to CNS abnormalities, alcohol exposure has been associated with heart abnormalities. Early observations indicated that alcohol exposure was associated with abnormal heart and great vessel (large vessels that bring blood to and from the heart) development following acute ethanol exposure in mice (Daft et al., 1986). Subsequently, prenatal alcohol exposure has been associated with altered myocardial contractile function, which may to contribute to the development of postnatal cardiac dysfunction (Ren et al., 2002). Furthermore, the aetiology of this cardiac dysfunction is thought to be due to increased intracellular Ca^{++} loading and apoptosis, as it was observed that elevated intracellular Ca^{++} levels in myocytes following prenatal alcohol exposure was associated with myocyte apoptosis. Heart defects have been associated with prenatal alcohol exposure, where cardiovascular malformations and congenital heart defects have been observed in the foetal alcohol syndrome (Burd et al., 2007; Steeg and Woolf, 1979).

2.4.2.2. DMRs are associated with immune response genes

Regulation of type I interferon production

Gene ontology biological processes identified from the SA model data that pertained to immunity included: *innate immune response*; *regulation of interferon beta (INF-β)*; *immune response*; and *regulation of type I interferon production*.

Type I interferons (IFNs) comprise a vast and growing group of IFN proteins. All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. Homologous molecules to type I IFNs are found in

many species, including all mammals. The mammalian types are designated IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega), and IFN-ζ (zeta, also known as limitin). The IFN-β proteins are produced in large quantities by fibroblasts. They have antiviral activity that is involved mainly in innate immune response. IFN-α and IFN-β are secreted by many cell types including lymphocytes (natural killer (NK) cells, B-cells and T-cells), macrophages, fibroblasts, endothelial cells, osteoblasts and others. They stimulate both macrophages and NK cells to elicit an anti-viral response, and are also active against tumours. Type I interferons activate intracellular antimicrobial programmes and influence the development of innate and adaptive immune responses. Canonical type I IFN signalling activates the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, leading to transcription of IFN-stimulated genes (ISGs). Host, pathogen and environmental factors regulate the responses of cells to this signalling pathway and thus calibrate host defences while limiting tissue damage and preventing autoimmunity (Ivashkiv and Donlin, 2014).

There is considerable evidence to suggest that epigenetic mechanisms, particularly DNA methylation, contribute to the regulation of immunity and immune response. As reviewed in Lim et al (2013) the T cell epigenome chromatin landscape has been characterised in terms of histone occupancy, histone modification patterns and transcription factor association with specific genomic regulatory regions. These chromatin modifications have been implicated in expression of key immune-responsive genes in response to T-cell activation. Histone modifications have also been associated with the epigenetic regulation of type I IFN-induced STAT activation and ISG transcription and translation. These regulatory mechanisms determine the biological outcomes of type I IFN responses and whether pathogens are cleared effectively or chronic infection or autoimmune disease ensue (Ivashkiv and Donlin, 2014). Micro RNAs have also been found to contribute to the regulation of immune

responses, particularly that of IFN-β innate immune response (Witwer et al., 2010). Cellular memory is a hallmark of adaptive immunity, whereby lymphocytes respond to antigens previously encountered by the immune system. Support for an epigenetic mechanism that regulates this memory stems from findings by Lai et al (2013), who profiled the B-lymphocyte methylome and transcriptome before and after antigen exposure using a human in vivo model. These authors observed significant activation-induced loss of DNA methylation at transcription factor binding sites and Alu element loci across the genome, which was concomitant with repression of DNA methyltransferase 3A. These epigenetic changes were retained in the progeny of activated B cells, and provide support for epigenetic activation and memory during cellular differentiation in an immune response. Furthermore, Makar et al (2003) and Lee (2001) demonstrated that thymocytes actively recruit DNA methylytransferases (particularly Dnmt1) during their maturation into T cells in the event of an immune response. More specifically, cell-type specific DNA methylation changes have been observed at intragenic CpG islands during cellular differentiation in the immune system (Deaton and Bird, 2011), wherein these DNA methylation changes are reported to be critical for the differentiation of stem cells into myeloid and lymphoid lineages, and thereby contributes to cellular plasticity and immune function (Suarez-Alvarez et al., 2012). Moreover, Fitzpatrick (1998) showed evidence that demethylation at the promoter region of *Interferon γ* (*IFN-γ*) and consequent mRNA expression in activated T cells, is heritable. These data therefore indicate the importance of IFN methylation during immune responses, and the transmission of an epigenetic memory of the immune system.

Immune deficiency is associated with alcohol exposure

Alcohol is known to enhance the risk of developing medical conditions related to immune system dysfunction. These include acute respiratory distress syndrome (ARDS), liver cancer, and alcoholic liver disease (ALD). Binge and chronic alcohol intake have also been associated

with increased susceptibility to pathogen infection, and advance the progression of HIV infection by weakening both the innate and adaptive immune systems (Curtis et al., 2013).

Alcohol's ability to dysregulate the innate immune response and influence inflammation responses have been extensively supported. Alcohol exposure has been shown to stimulate the hypothalamus to produce corticotropin-releasing hormone (CRH), which triggers the pituitary gland to secrete adrenal corticotropic hormone (ACTH), which in turn stimulates the adrenal glands to release glucocorticoids. When expressed at high levels, glucocorticoids are able to influence the production of cytokines from white bloods cells, which is able to supress inflammatory and immune responses (Kovacs and Messingham, 2002). Similar findings of alcohol's ability to influence immunity and inflammation have been supported by subsequent studies (Boe et al., 2010; Brown et al., 2006; Goral et al., 2008; Nagy, 2003; Waldschmidt et al., 2008).

More recently, the ability for alcohol to affect the immunity of specific organs has been demonstrated. Using a C57BL/6 mouse model, Osterndorff-Kahanek et al (2013) showed that gene expression changes in both the brain (prefrontal cortex) and liver were evident in mice which were chronically exposed to ethanol. The changes in gene expression as a consequence of alcohol exposure were similar to those observed in mice only exposed to lipopolysaccharide (LPS) – a strong immune response elicitor. These authors concluded that chronic alcohol consumption is associated with immune activation. These findings are also supported by Kane et al (2013), who demonstrated region-specific changes in chemokine and cytokine expression in the brain as a result of ethanol exposure (6g/kg) of C57BL/6 mice. Expression profiles of the hippocampus, cerebellum and cerebral cortex showed region-specific susceptibility to alcohol and the regulation of neuroinflammation.

Foetal exposure to alcohol has also been shown to affect immunity. Alcohol use during pregnancy has been shown to increase neonatal risk of infection by 2.5 fold (Johnson et al., 1981) and excessive alcohol abuse is associated with new-borns who are 3–4 times more likely to acquire severe neonatal infections (Gauthier et al., 2005). Immune deficiency has been associated with foetal alcohol syndrome, where cases of increased incidence of minor and life-threatening bacterial infections were observed in 13 documented cases of FAS (Chiappelli and Taylor, 1995; Johnson et al., 1981). Furthermore, exposure to ethanol during the last trimester of pregnancy has been shown to alter the maturation and immunity of the foetal lung (Lazic et al., 2011). Further to this, alcohol is known to impact on the neuroimmune system in the developing central nervous system, which contributes to FASD (Kane et al., 2012). There is also evidence to suggest that the effect of foetal alcohol exposure is associated with increased susceptibility to infection in adulthood in mice (McGill et al., 2009).

Overall, there is considerable evidence to suggest that epigenetic mechanisms, particularly DNA methylation, contribute to the regulation of immunity and immune response. It has been proposed that alcohol exposure alters DNA methylation of cells of the immune system, which subsequently (dys)regulates immune response (Curtis et al., 2013). Furthermore, FAS has been associated with immune deficiencies and consequent increases in infections in humans. The aetiology of this may therefore be explained by a similar mechanism proposed above. That is, ethanol exposure in utero is able to alter the epigenetic state of foetal genes responsible for regulating the immune system. As shown by Fitzpatrick et al (1998), this altered state may be somatically inherited (inherited through subsequent cellular division) and consequently alter immunity in offspring. From this (although this study did not quantify DNA methylation within the foetal epigenome of embryos sired by ethanol-exposed males) it

may be plausible that altered epigenetic states of genes that regulate immunity contained within the sperm of ethanol exposed males are inherited by sired offspring.

2.4.2.3. Glycoprotein metabolism may be linked to neural development and immune function

Gene ontology analyses from the SA and CAM models, identified biological processes pertaining to glycoprotein metabolism, neural development and immune function (Table 10). Glycoproteins are proteins that contain oligosaccharide chains (glycans) attached to polypeptide side-chains. Glycoproteins commonly occur as integral membrane proteins, which enable cell-cell interactions (Takahashi et al., 2009). Further to this, glycoproteins have been shown to play a pivotal role in fertilisation, neuronal development, immune surveillance and inflammatory responses (Gamblin et al., 2009). It is therefore plausible that glycoprotein metabolism, at least in part, regulates neural development and immune function.

Glycoproteins and neural development

Nel (neural epidermal growth factor (EGF)-like molecule) is a multimeric, multimodular extracellular glycoprotein with heparin-binding activity and structural similarities to thrombospondin-1. Nel is predominantly expressed in the nervous system and has been implicated in neuronal proliferation and differentiation, retinal axon guidance, synaptic functions, and spatial learning (Nakamura et al., 2012). 5T4 glycoprotein contributes in the regulation of activity-dependent dendritic development of interneurons and the formation of functional neural circuitry in the olfactory bulb (Miyasaka et al., 2013). Synapsin I is an oligomannose-binding lectin and glycoprotein, that can be released from glial-derived

exosomes and promotes neurite outgrowth and neuronal survival by modulating the interactions between glia and neurons (Wang et al., 2011).

Glycoprotein involvement in the immune system and inflammation

All immune receptors localised on the cell surface are glycoproteins. These include major histocompatibility complex proteins (MHC class I and MHC class II) and T and B cell receptors and co-receptors (Rabinovich et al., 2012). The glycans contained within glycoproteins also play a role in inflammation. It has been noted that remodelling of cell surface glycans plays a role in the transition from normal to inflamed tissues (Dube and Bertozzi, 2005). These changes mediate emigration and trafficking of immune cells to sites of inflammation (Sperandio et al., 2009).

The effect of alcohol on glycoproteins

Alcohol exposure has been associated with several changes to glycoproteins. Sun and Seaman (1977) demonstrated increased Ca^{++} uptake in synaptosomes from animals chronically administered ethanol. It was later observed that increased uptake of calcium at synaptic plasma membranes was associated with increased binding of sialic acid membrane glycoproteins (Ross et al., 1977). Transferrin is an iron-binding blood plasma glycoprotein that regulates the level of free iron in biological fluids (Crichton and Ward, 1992). Alcohol exposure, and subsequent metabolism, has been associated with acetaldehyde-mediated inhibition of glycosyl transfer to transferrin (Enomoto et al., 1991; Stibler and Borg, 1991). More recently, Gravel et al (1996) revealed that alcoholic and cirrhotic patients show alterations of serum glycoproteins secreted by the liver.

Interestingly, chronic maternal ethanol consumption prior to parturition has been shown to elicit an effect on the synthesis of synaptic plasma glycoproteins in developing offspring (Noronha and Druse, 1982). This finding has been supported by Zoeller et al (1994), who

observed that in utero ethanol exposure during a period of rapid myelination alters the expression of mRNAs encoding Myelin Basic Protein and Myelin-Associated Glycoprotein in the cerebellum of 15 day old pups.

2.4.2.4. DMRs are associated with genes that regulate locomotor activity

GO analysis of both models revealed enrichment for regulation of locomotor activity, but only in the SA model. This GO process included genes *Htr2c*, *Tbx5*, and *Cxcl12*. *Htr2c* and *Tbx5* were significantly hypermethylated ($p<0.01$), while *Cxcl12* was hypomethylated ($p<0.01$), relative to control sperm DNA methylation levels.

The understanding of human motor development have moved from the pure maturation of predetermined patterns of the central nervous system, to one by which the phenotype develops through a complex interaction of genetic and environmental components, that is, epigenetic systems (Forssberg, 1999).

The development of foetal movements begins with slow extensions of the neck during the seventh gestational week. This progresses to general movements, breathing, jaw, rhythmical sucking, and swallowing movements (Prechtl et al., 1997). These early movements are patterned and continue almost unchanged in form and shape after birth. At birth, neonatal movements include feeding, respiration, blinking coughing, masticating and swallowing (Forssberg, 1999). Because these innate foetal and infantile movements remain relatively unchanged, it is likely that they are generated by similar types of epigenetic central networks (Forssberg, 1999).

As early as 1973, the effects of environmental exposures (X-rays) to the brain during infancy were observed to negatively affect cellular development (retardation) which resulted in locomotor deficits in adult rats (Brunner and Altman, 1973). Subsequent studies using animal models confirmed the effect of in utero alcohol exposure on foetal locomotion. Prenatal alcohol exposure of C57BL/6 mice affected locomotor activity and passive avoidance behaviour (Becker and Randall, 1989), and in guinea pigs, prenatal alcohol exposure was associated with hyperactivity, and decreased brain and hippocampal weight without a change in postnatal body weight (Gibson et al., 2000). Using a "third trimester equivalent" rat model (exposure at postnatal days 4-9) Goodlett and Lundahl (1996) demonstrated that alcohol exposure induced severe neonatal Purkinje cell loss and motor performance deficits. Thomas et al (1998) further demonstrated that alcohol-induced Purkinje cell loss with correlated motor performance deficits is dependent on developmental timing of exposure.

Using a Drosophila model of FAS, McLure et al (2011) showed that in utero alcohol exposure resulted in increased locomotor activity, which is supported by Sanchez Vega et al (2013) who demonstrated that early gestational exposure to moderate concentrations of ethanol alters adult behaviour in C57BL/6J mice. The main findings were that mice had persistent and long lasting alterations in behaviour, including hyperactivity and enhanced spatial memory. These data suggest that even moderate dose ethanol exposure in early gestation has long term consequences on brain function and behaviour in mice.

Foetal alcohol syndrome in humans is associated with cognitive impairment in various neuropsychological domains, including overall intellectual performance, executive function, learning and memory, language, visual-spatial ability, behavioural problems, motor function, attention, and activity levels (Mattson et al., 2011). FASD studies have demonstrated that prenatal alcohol exposure has been associated with a spectrum of motor performance deficits. These include poor hand/eye coordination, delayed motor reaction timing, tremors,

dysfunctional force regulation, and atypical trajectories in goal-directed arm movements. These deficits affect both gross and fine motor coordination (CDC, 2004). These deficits are likely to be attributable to atypical muscle movement, reduced motor neurons, poor peripheral myelination, and slowed nerve conductivity. While some deficits normalise with age, most persist into adulthood.

Our study observed enrichment for biological processes related to the regulation of locomotion in the significant DMRs of sperm from mice chronically exposed to alcohol in the SA model. However, this was not observed in the GO analysis of the CAM model. Although these models were virtually identical in design, the SA model exposed male mice to alcohol for a period that was more than double than of the CAM model (10 weeks v. 27 weeks). It is widely accepted that the volume and duration of prenatal alcohol exposure impacts on the severity of foetal alcohol effects, with lengthier exposure durations generally associated with more severe phenotypes (CDC, 2004; Smith et al., 1986). FASD encompasses the following spectrum disorders (in order of severity): alcohol-related birth defects (ARBD), foetal alcohol effects (FAE), alcohol-related neurodevelopmental disorder (ARND), partial FAS (pFAS) and foetal alcohol syndrome (FAS). While all of these spectrum disorders are associated with neurodevelopmental deficits (to varying degrees), it is only the most severe of these disorders that tend to manifest with growth deficiencies (pFAS and FAS) (Astley, 2004) and locomotor dysfunction (FAS) (CDC, 2004). This observation may explain the findings of our study, where we observed enrichment for neural development in both our models, but only enrichment for regulation of locomotor activity in our SA model, and not in our CAM model. That is, due to the longer period of exposure in our SA model (27 weeks), this model may be more alike that associated with FAS, while that of our CAM model, akin to that of a FAE/ARND/pFAS model.

2.4.2.5. DMRs are associated with genes that regulate sexual reproduction

The effect of environmental exposures on male gametes has received considerable attention. Chemicals capable of disrupting reproductive function in both humans and animals have been documented (Perry, 2008). It has been estimated that exposure to environmental factors account for 40% of deaths globally (Pimentel et al., 2007), which are attributed to differences in lifestyle, exposure to dietary compounds, and exposure to environmental toxicants. These influences are likely to account for the increasing incidence of abnormalities of the male reproductive system (Giwercman and Giwercman, 2011).

In particular, the direct exposure to pesticides can impact on spermatogenesis and cause infertility. These exposures are usually effective through hormonal or genotoxic pathways, where they can cause gross sperm morphology aberrations, numerical chromosomal aberrations, fragment DNA, and affect vitality and mobility. Studies on occupational exposure to pesticides have been associated with male infertility, testicular dysfunction and abnormalities (Kumar et al., 2000). Endocrine-disrupting chemicals have been associated with reproductive disorders in wildlife, including sex reversal, impaired viability of offspring and changes in socio-sexual behaviour (Fox, 2001).

Environmental epigenetics is used to describe the ability of environmental factors to influence epigenetic marks, which can consequently alter gene expression and elicit an aberrant phenotype, including disease states and subfertility (Inbar-Feigenberg et al., 2013; Skinner et al., 2010). Vinclozolin is a common fungicide, which disrupts endocrine function in mammals. Exposure of male rats to vinclozolin has been associated with decreased prostate weight, weight reduction in sex organs, abnormal nipple development, decreased anogenital distance, and sex organ malformations. Embryonic exposure to vinclozolin can influence sexual differentiation, gonadal formation, and reproductive functions (Anway and Skinner,

2006). An epigenetic mechanism for vinclozolin-mediated endocrine disruption was proposed (Anway, 2005) and has received much support (Anway and Skinner, 2006; Guerrero-Bosagna et al., 2010). Vinclozolin has been shown to induce epigenetic aberrations in DNA methylation of promoter regions in sperm, which can be transgenerationally inherited in a sex-specific manner to the F3 generation (Guerrero-Bosagna et al., 2010) and is associated with reproductive abnormalities (sperm count and sperm motility) (Anway, 2005; Kelce et al., 1994).

Similar to the effects of vinclozolin and pesticides, acute intake of excessive alcohol during puberty has been shown to induce sexually dimorphic changes in genes regulating the hypothalamo-pituitary-adrenal (HPA) axis (Przybycien-Szymanska et al., 2010). The HPA axis is stimulated by the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which in turn causes the release of adrenal glucocorticoids. As discussed previously, alcohol consumption is able to stimulate the hypothalamus to produce corticotropin-releasing hormone (CRH), which ultimately stimulates the adrenal glands to release glucocorticoids. In utero alcohol exposure has been associated with testicular atrophy (Horrobin, 1980) as well as epimutations in sperm DNA methylation, which are transgenerationally transmissible (Stouder et al., 2011).

From the above, it is highlighted that the germline is capable of (if not responsible for) transmitting epigenomic information to subsequent generations. It consequently follows, that if altered by an environmental exposure (such as alcohol), the germline may be able to transmit incorrect epigenetic information which could subsequently impact on the gene expression and phenotype of subsequent generations.

2.4.2.6. DMRs are associated with cell proliferation, cell motility, and cellular cytoskeleton

The involvement of DNA methylation in regulating cell motility and proliferation is derived primarily from cancer research. Aberrations of fibroblast growth factors (FGF) and their receptors have been shown to contribute to the progression of prostate cancer by enhancing cellular proliferation, survival and motility. Wang et al (2006) observed that Sprouty4, a negative regulator of FGF signalling, is downregulated in human prostate cancer by DNA methylation, which was though to contribute to enhanced cell migration. The micro RNA, miR-34b, has been shown to be hypermethylated in melanomas, but not in melanocytes or keratinocytes. Expression of miR-34b has been associated with reduced cell invasion and motility rates in melanoma cell lines (Mazar et al., 2011). From this observation, these authors concluded that reduced expression of miR-34b is associated with enhanced cell invasiveness and motility in metastatic melanoma cells. *RASSF1A* is a tumour suppressor gene that plays a role in various biological processes, including cell growth, differentiation, apoptosis, and cell motility (Vos et al., 2000). The *RASSF1A* promoter is generally hypermethylated in a variety cancer types, including lung and pancreatic cancers (Dammann et al., 2003). Mengxi et al (2013) recently demonstrated that reversal of *RASSF1A*'s promoter methylation status using 5-Aza-2′-deoxycytidine (a DNA methyltransferase inhibitor) resulted in increased apoptosis of A549DDP cells in culture and a concomitant block in cell motility in the G0/G1 stage. In an attempt to investigate the mechanism by which haematopoietic stems cells lose functional potential during aging, Beerman et al (2013) demonstrated that the epigenome of aged murine haematopoietic stem cells undergo alterations in DNA methylation, which was associated with a decline in proliferative potential. Together, these lines of evidence suggest that cellular motility and proliferation are, at least in part, regulated by DNA methylation.

Alcohol exposure has been shown to negatively impact on cellular migration, proliferation and acquisition of epigenetic marks. Prenatal alcohol exposure has been shown to alter the migration of cortical neurons (Miller, 1993) and the laminar distribution of callosal projection neurons (Miller, 1997), which has been associated with defects in neuronal migration (Kotkoskie and Norton, 1988). In particular, prenatal alcohol exposure has been shown to retard the migration and development of serotonin neurons in mouse foetuses (Zhou et al., 2001), and inhibit the proliferation of glioma cells (Mikami et al., 1997). The aetiology of these defects has been associated with DNA methylation aberrations. Zhou et al (2011a) demonstrated that exposing neural stem cells in culture to alcohol alters DNA methylation patterns and inhibits neural stem cell differentiation. Moreover, alcohol exposure prevented the establishment of DNA methylation of specific genes associated with neural development, eye development, and developmental disorders.

The regulation of cell adhesion and migration is essential for embryonic development, immune response and wound healing (Mayanagi and Sobue, 2011). These processes are also involved in pathological events, including cancer progression and mental retardation (Wang et al., 2005). During cell migration, cells dissociate from their origin and relocate to distant tissues. The process of cell adhesion and migration are controlled by changes in the cytoskeleton, particularly the actin cytoskeleton (Pantaloni et al., 2001). The actin cytoskeleton regulates cell motility, adhesion and migration (Chhabra and Higgs, 2007).

Prenatal alcohol exospore in animal models has been associated with a variety of pathologies involving the cellular cytoskeleton (Evrard and Brusco, 2011). Muscle biopsies obtained from rat pups exposed to ethanol *in utero* demonstrated disorganisation of myocyte cytoskeleton (Adickes and Mollner, 1986). Using a similar model, Saez et al (1991) observed disorganisation of the cytoskeleton of astrocytes obtained from rats prenatally exposed to ethanol, which was associated with decreased cytoskeletal proteins. Other studies have

observed cytoskeletal dysfunction of the developing intestinal epithelium (Montes et al., 1996) and hepatocytes (which was associated with glycoprotein heterogeneity) (Azorin et al., 2004) of rats prenatally exposed to alcohol, as well as hippocampal neurons exposed to alcohol in culture (Romero et al., 2010). Using a zebrafish model, Sarmah et al (2013) demonstrated that embryo cultures exposed to ethanol induced gastrulation defects where they observed microtubule cytoskeleton disruption and blastomere adhesion defects. An epigenetic mechanism that may underlie these cytoskeleton pathologies has recently been investigated. Using a genome-wide approach, Hughes et al (2014) identified altered DNA methylation among cytoskeletal remodelling genes in monocytes and CD4+ T cells in Behçet's disease. They identified 383 monocyte differentially methylated CpG sites between Behçet's disease patients when compared to controls. Bioinformatic analysis of these genes identified multiple classes of structural and regulatory proteins of the cytoskeleton.

Together, these findings show that prenatal ethanol exposure is associated with defects in cell motility, proliferation and cytoskeleton, which may be caused by a disruption in DNA methylation.

2.4.3. Chronic alcohol exposure of male gametes does not result in hemi-methylated DNA

The effect of alcohol on biological systems is numerous. Among these is alcohol's apparent ability to impact on *DNA methyltransferase* (*Dnmt*) expression. In utero alcohol exposure in mice has been associated with decreased methyltransferase activity and concurrent loss of foetal DNA methylation (Garro et al., 1991), while alcoholism in humans has been associated with significant decreases in *DNMT3a* and *DNMT3b* expression, but no change in *DNMT1* expression (Bonsch et al., 2006). Male rodents exposed to alcohol for nine weeks before

breeding was associated with decrease DNA methyltransferase mRNA levels in paternal sperm (Bielawski et al., 2002). Subsequent study by Zhou et al attempted to understand the effects of alcohol exposure on epigenetic mechanisms and the consequences it has on the CNS. Their first study revealed that alcohol exposure prevents the establishment of normal DNA methylation patterns in neural stem cells genes which then has implications for their differentiation, which was associated with both an increase (hypermethylation) and decrease (hypomethylation) in DNA methylation (Zhou et al., 2011b). They then went on to find that epigenetic reprogramming during neural development is altered by alcohol and that the inhibition of epigenetics at the same stage mimics the alcohol induced growth retardation in the face and organs such as the brain and heart (Zhou et al., 2011b). From this, these authors concluded that DNA methylation plays a role in the aetiology of FAS, where abnormalities of the brain are hallmarks of the syndrome.

As discussed previously, Dnmt1 is a maintenance DNA methyltransferase. That is, Dnmt1 faithfully replicates existing DNA methylation signatures during DNA replication, ensuring symmetrical DNA methylation marks are established on daughter strands, using the parental strand as a template. Dnmt1 has been implicated in the sperm DNA methylation aberrations observed following preconception paternal alcohol exposure. Using an in utero mouse alcohol exposure model, Govorko et al (2012) noted significant deficit in POMC neural function, which was associated with aberrant hypermethylation of CpG sites contained within the *Pomc* promoter, as well as altered levels of Dnmt1 in POMC neurons. Furthermore, they observed that this epigenetic aberration was transgenerationally transmitted through the male germline. These authors concluded that Dnmt1 has a possible role in foetal alcohol-induced changes in *Pomc* gene methylation and expression. Similarly, Warnault et al (2013) demonstrated that excessive alcohol intake increases DNMT1 levels and reduces histone H4 acetylation in the nucleus accumbens area of the brain in rodents. In

contrast to these findings, Kutay et al (2012) demonstrated that exposing wild type *Dnmt1* mice to ethanol for six weeks caused a pronounced decrease in Dnmt1 activity in the liver of treated mice. In support of this finding, clinical studies have shown that post-mortem human alcoholic brains show reduced DNA methylation and DNMT1 levels in the amygdala and the superior frontal cortex (Ponomarev et al., 2012). Despite these opposing findings, these studies do suggest that alcohol is able to alter Dnmt1 activity (whether directly at the protein level or mRNA level).

From the above, the current study therefore hypothesised that chronic preconception paternal alcohol exposure would impact Dnmt1 activity, and consequently abrogate its maintenance activity, which in turn would cause asymmetrical DNA methylation signatures, and increase the incidence of hemi-methylated DNA strands within the sperm epigenome of alcohol exposed mice. However, our data did not demonstrate this, as we observed a non-significant difference between the two treatment groups (p=0.548). From this, it can be concluded that ethanol exposure to sperm DNA does not affect maintenance DNA methylation during spermatogenesis.

Despite the insignificant difference in hemi-methylation between the two treatment groups, a greater degree of variance in levels of hemi-methylation within the ethanol treated group, when compared to that of the sucrose treated control group was observed (Figure 31). In the ETOH group, the number of hemi-methylated sites ranged from 266 to 850; while that of the CTRL group ranged from 502 to 596. However, the lower limit of the number of hemi-methylated sites in the ETOH group was derived from the rate-limiting dataset of ETOH1. Thus, conservatively (removing ETOH1), the range observed in the ETOH group was 539 to 850. This observation might imply that subtle increases in the number of hemi-methylated DNA sites are evident in the ethanol exposed group, and may suggest a nuanced effect of alcohol on levels of hemi-methylated DNA, possibly via abnormal Dnmt1 activity during

spermatogenesis (successive DNA replication cycles), and that statistical significance was not reached as a consequence of a small sample size (ETOH n=4 and CTRL n=5).

2.4.4. Chronic paternal preconception alcohol exposure induces epimutations at CGIs which may be transmissible

Overall, our DNA methylation data obtained from the sperm of male mice exposed to alcohol prior to conception confirms that chronic exposure is able to alter (increase and decrease) DNA methylation at CpG islands (CGIs) of the male gamete epigenome, but which are not associated with ICRs. This was primarily evident at regions of low methylation (LMRs), which are reported to be more sensitive to environmental exposures, and which are important in embryonic development. Further to this, the presence of these aberrant DNA methylation signatures in the mature sperm indicates that DNA methylation errors were not corrected during spermatogenesis and sperm maturation.

With support of findings by Govorko et al (2012) and Manikkan (2012), it might then be plausible to draw parallels between direct prenatal exposure models with the indirect paternal preconception exposure model of the current study, in as far as alcohol-induced epigenetic changes are transmissible and consequently impact on phenotypic outcome in offspring. That is, paternal environmental exposures that alter sperm DNA methylation (such as alcohol), which are not corrected during spermatogenesis and sperm maturation, could be transmitted to sired offspring, escape epigenetic reprogramming in the zygote, and consequently alter gene expression, and consequently elicit an aberrant phenotype that is synonymous with FAS.

Chapter 3

The effect of preconception paternal alcohol exposure on offspring outcome

3.1. Introduction

Studies demonstrating the effects of the parental environment on offspring outcome have predominantly focused on maternal transmission of disease states. Studies using rodent models have demonstrated that photoperiod information can be transmitted to offspring following *in utero* exposure to specified periods of light (as described in Horton, 2005); stress reactivity and grooming behaviour can be maternally inherited (Meaney et al., 2007; Weaver et al., 2004); and adverse foetal environments have been shown to be associated with an increased risk of cardiovascular, metabolic, neuroendocrine, and psychological disorders in adulthood (as reviewed in Harris and Seckl, 2011).

Maternal consumption of alcohol during pregnancy has been associated with foetal alcohol syndrome. Foetal alcohol syndrome does not manifest in every individual who is exposed to alcohol *in utero*, and the degree to which individuals are affected, differs (Nayak and Murthy, 2008). The contributing factors to the variations seen may include maternal drinking patterns, genetic susceptibility, a difference in the maternal metabolism of alcohol, and variations in the vulnerability of different regions of the brain (Nayak and Murthy, 2008). Alcohol's many effects on an organism, including the brain, result in cell death. Evidence of the pathogenic mechanisms comes primarily from animal models (Mukherjee et al., 2006). These models showed that the cell populations and affected brain regions differ depending

on the developmental stage during ethanol exposure. Although the effects of ethanol have a crucial impact on various cellular activities, evidence suggests that a critical pathogenic feature associated with ethanol induced malformations is cell death (Dunty et al., 2001).

The two general processes of cell death that exist include necrosis and apoptosis. These processes are differentiated by distinct cellular morphological and biochemical changes during cell death. Necrosis of the nervous system occurs when neurons become damaged as a result of an insult (environmental, mechanical or metabolic injury) and typically involves the concurrent death of adjacent cells (Goodlett and Horn, 2001). During the necrotic process, cells swell and their organelles break down. This eventually leads to cellular rupture and the release of cell debris. This consequently leads to local inflammation. In contrast to this, apoptosis only affects individual cells. During this process, the cell body shrinks, and the DNA contained within the nucleus condensed before breaking apart into smaller fragments. These fragments are then engulfed by scavenging cells (Goodlett and Horn, 2001).

Several factors have been shown to induce apoptosis of the CNS. These include insufficient blood supply to the brain, dysfunction of cellular energy generation, disruption of normal cellular calcium levels, and oxidative stress.

Programmed cell death (PCD) is a genetically controlled and highly regulated pathway that occurs in the developing embryo, and deviation from this pattern may lead to dysmorphogenesis (Dunty et al., 2001). Apoptosis is a form of PCD and accounts for most cell death (as opposed to necrosis which is the death of a cell or tissue caused by external factors such as trauma or infection). Apoptotic neurodegeneration is triggered by ethanol through a twofold mechanism: blockade of the N-methyl D-aspartate (NDMA) glutamate receptors and excessive activation of gamma-aminobutyric acid (GABA) receptors (Dunty et al., 2001). The key mechanism behind the typical facial features and CNS abnormalities that develop

simultaneously in FAS is cellular death at the rostral boundary of the preclosure forebrain and the corresponding cell population involved in the postclosure telencephalic midline (Dunty et al., 2001). This population is now defined at the anterior neural ridge (ANR) and plays a role during gastrulation and early gastrulation stages as a coordinator for the prosencephalon (Dunty et al., 2001). There are multiple cell lines that are sensitive to ethanol-induced cell death. These populations include: the epithelium that lines the nasal cavities, the neural crest, epibranchial placodes and subpopulations of the optic placodes or vesicles (Dunty et al., 2001). In support of these findings, Sari et al (2013) demonstrated that daily intraperitoneal injection of alcohol during E7-15 in the mouse resulted in significantly increased apoptosis in the primordium of the cerebral cortex and reduced brain weight. More recently, Chen et al (2013) showed that DNA methylation programming proceeds along with hippocampal neuronal differentiation and maturation, and that prenatal exposure to ethanol from E7-16 negatively impacted on the acquisition of both 5mC and 5hmC in the hippocampus, which was associated with developmental retardation.

Different patterns of neuronal loss are a consequence of the timing of ethanol exposure during either early, mid or late phase of synaptogenesis with each having the ability to cause distinctive neurobehavioral disturbances (Nayak and Murthy, 2008). A mouse model study revealed that ethanol exposure during gestational day seven, that is equivalent to the third week in a human pregnancy leads to deficiency of the anterior midline brain structure (as reviwed by Kotch and Sulik, 1992). However ethanol exposure during day eight involves loss off premigratory cranial neural cell populations. Ethanol exposure through the second half of the rodent gestation disturbs the pyramidal cells in the hippocampus and reduces the neurons of the trigeminal nerve (as reviewed by Kotch and Sulik, 1992). Neuronal populations appear to be the most sensitive to ethanol exposure. This demonstrates the direct cytotoxic effects of alcohol.

Alcohol has many actions on the developing organism, particularly that of the brain, where it is able to induce cell death. The processes of induced cell death include necrosis and apoptosis. While apoptosis affects single cells, the debris released from the necrotic process leads to death of adjacent cells and localised inflammation. Apoptosis of the CNS can be induced through insufficient blood supply to the brain, dysfunction of cellular energy, disruption of normal cellular calcium levels, and oxidative stress (Goodlett and Horn, 2001). Further to alcohol's ability to induced cell death of the CNS, alcohol is able to induce physiological effects on the liver, endocrine system, and bone density. Effects on the liver include altered enzyme levels which are indicative of liver damage in humans (Clark et al., 2001). Effects on the endocrine system include lower oestrogen levels in adolescent girls (Block et al., 1993) and lowered luteinising hormones in adolescent boys (Frias et al., 2000); adverse effects on maturation of the female reproductive system following alcohol exposure during adolescence in rats (Dees et al., 2001); delayed puberty in female rats as a result of suppression of reproductive hormones (Emanuele et al., 2002); disrupted regulatory systems within the ovaries (Dees et al., 2001); and altered growth hormone and testosterone levels in male rats (Cicero et al., 1990; Emanuele et al., 1999; Little et al., 1992; Steiner et al., 1997). Alcohol exposure has been shown to be associated with lowered bone density in human adolescent males, but not females (Elgan et al., 2002; Fehily et al., 1992; Fujita et al., 1999; Neville et al., 2002); and shorter limb lengths and reduced bone growth in rats (Sampson et al., 1999; Wezeman et al., 1999).

As indicated above, the clinical consequences of *in utero* ethanol exposure are highly variable and it has therefore been necessary for researchers to approach alcohol teratogenesis from multiple angles such as genetic, biochemical, cellular and morphological (as reviewed by Haycock, 2009). Although considerable efforts have been made to elucidate the aetiology of FAS at these levels, the fact that paternal contributions to FAS have been

reported, suggests that ethanol can act indirectly to elicit a FAS-like phenotype. In light of research in recent years it is not uncommon to suggest that paternal environmental influences can alter the phenotype of future generations.

3.1.1. Phenotypes associated with paternal environmental exposure

More recently, studies have demonstrated that environmental exposures of fathers prior to conception can influence the outcome of their offspring. Rodent models have shown an association between paternal low protein diet and hepatic function in offspring (Carone et al., 2010), and paternal chronic exposure to HFD affects β-cell function in F_1 female offspring (Ng et al., 2010). Combined with the study showing that offspring glucose levels are affected by preconception paternal fasting in mice (Anderson et al., 2006) these results demonstrate that in rodents, paternal diet exerts wide-ranging effects on metabolism in the offspring.

A variety of phenotypic outcomes in offspring have been associated with paternal alcohol use. These include immune system dysfunction (Gottesfeld and Abel, 1991), altered grooming behaviour (Abel, 1991b), increased foetal weight (Abel, 1995), gross malformations (Bielawski and Abel, 1997; Stockard and Papanicolaou, 1916), increased spleen weights (Abel, 1993b), decreased infant birth weight (Little and Sing, 1987), decreased litter size, decreased testosterone levels, and altered offspring activity (Abel, 2004; Abel, 1989; Abel, 1991a; Abel, 1993a; Abel, 1993b; Friedler, 1996), including hyperactivity (Abel, 1995; Niewald et al., 1998). These findings demonstrate that the effect of alcohol is not consistent across studies, and likely reflect the influence of varying length and timing of exposure, as well as genetic backgrounds in animal models.

3.1.2. Paternal contributions to foetal alcohol syndrome

Although FAS research has been primarily focused on maternal drinking during pregnancy, it is surprising to note that there is substantial and compelling evidence of a paternal contribution. Various studies have been directed toward understanding the paternal effects of prenatal alcohol exposure. As early as 1621, Gellius in the *Anatomy of Melancholy* noted that "if a drunken man get a child, it will never likely have a good brain" (as stated in Friedler, 1996), that is, it was observed that children of alcoholic men had "weak" brains. It is of significant interest to note that even before the potential adverse effects of excessive paternal alcohol consumption were formally acknowledged, in 1911, Forel cites an account by Schweighofer where a woman marries a man, neither of whom abused alcohol, and have three children of good health. The man dies and the woman re-marries a drunkard. She bares three more children: one became a drunkard; another had "infantilism"; and the third was a social degenerate and a drunk. Two of these children also had tuberculosis, which hadn't been seen in the family. The woman then marries a third time to a sober man, and produces children of good health once again.

The potential contribution of the father to FASD aetiology has remained of interest to researchers and is receiving increased attention. Lemoine et al. (1968) reported the first case of infants born with characteristic FASD symptoms to mothers who had not consumed alcohol during pregnancy, but whose fathers were alcoholics (Lemoine et al., 1968). It was later noted that 75% of children with FAS had biological fathers who were heavy drinkers or alcoholics (Abel et al., 1983). Following this finding, it was noted that sons of alcoholic fathers demonstrated cognitive impairment (Hegedus et al., 1984) and an association was reported in humans between father's drinking prior to conception and decreased infant birth weight (Little and Sing, 1987). It had been demonstrated that children born to alcoholic

fathers exhibited hyperactivity and reduced cognitive performance (Hegedus et al., 1984; Tarter et al., 1989).

Research on the influence of alcohol on mammalian offspring began in 1888, where Mairet and Cambemale (as described in Stockard, 1913) treated a dog with absinthe, and mated it with a normal bitch. She gave birth to twelve pups – two of which were still-born, three died after 14 days, and the rest died later due to intestinal complications and tuberculosis. In 1916 Charles Stockard (later joined by George Papanicolaou) made some fascinating observations about the transmission of deformities in descendants of alcoholised male guinea pigs (Stockard and Papanicolaou, 1916). For over seven years, their research tracked the development of offspring sired by males exposed to alcohol fumes through successive generations. They noted that the effect of alcohol on the male was of such a nature that an exposed male almost invariably "begets defective offspring even when mated with a vigorous normal female" (Stockard and Papanicolaou, 1916). These defects primarily involved the central nervous system and special sense organs, including opaque lenses and cornea of the eye, and in some cases even complete absence of eyes. Other abnormalities included low birth weights and limb deformities. Subsequently, experimentation and reports in the 1970s exposed the adverse effects of not only maternal, but paternal alcohol consumption on their offspring (Sulik et al., 1981). In support of the above, Lee et al (2013) observed a transgenerational effect of paternal alcohol exposure in mouse offspring. In this study, transgenerational toxicities in offspring, both in foetal and postnatal stages with paternal exposure to various alcohol concentrations was investigated. The exposure of these sires was associated with foetal agenesis and skull malformation (exencephaly). It was further observed that paternal alcohol exposure affected major organ weights of postnatal offspring. These authors concluded that paternal alcohol consumption prior to conception represents a potential risk to foetal and postnatal development.

Paternal alcohol use prior to conception has primarily been investigated in rat and mouse models. These studies have observed reduced birth weight, compromised immunity, behavioural deficits such as anxiety and aggressive like behaviour, reduced litter size, developmental retardation, increase susceptibility to infections and increased mortality in offspring sired to alcohol-exposed males (as reviewed by Curley et al., 2010).

This evidence opened the prospect that a number of FAS cases attributed to maternal alcohol abuse during pregnancy may have in fact been a result or partly due to excessive paternal drinking (Abel, 2004). The possibility of paternal preconception alcohol abuse on foetal development prompted further investigation, and studies using rat models found evidence that paternal exposure to alcohol had significant effects on the neurobehaviour of subsequent offspring (Jamerson et al., 2004). Others have reported congenital malformations, low birth weights, growth retardation and neonatal mortality associated with preconception paternal alcohol intake (Friedler, 1996), providing convincing evidence for the potential for paternally mediated foetal alcohol effects.

In our current study, we examined the effect of preconception paternal alcohol exposure on embryonic outcome. It was hypothesised that chronic alcohol exposure of male mice prior to conception, would induce epimutations in the sperm of alcohol exposed males, which would be transmitted to sired offspring and consequently impact on gene expression and embryonic development. Specifically, we postulated that a change in embryonic gene expression would manifest as a growth restricted phenotype. We therefore assessed the weights of embryos sired by ethanol treated males and compared this to those of embryos sired by sucrose exposed males as an indicator for overall success of foetal development. Tissue (organ) weight was used as an overall measure of embryonic growth and development. These three tissues were examined, as they are most commonly affected in

foetal alcohol syndrome Further to this, we quantified global gene expression in these tissues, in order to determine whether chronic alcohol exposure prior to conception elicits a change in gene expression.

3.2. Materials & Methodology

In Chapter 2, DNA methylation at CpG islands contained within the sperm genome was quantified using RRBS in order to assess whether chronic alcohol exposure negatively impacts on DNA methylation signatures of the sperm methylome. In Chapter 3, we investigated whether this chronic paternal alcohol exposure prior to conception impacts on the embryonic phenotype and gene expression in tissues commonly affected in foetal alcohol syndrome. Embryos were only generated from the CAM model.

To obtain embryos from treated sires, each treated male (numbered 1 – 24) was paired with two females – designated female R (identified by a right ear punch) and female L (identified by a left ear punch). Mating occurred over a period of one week. Insemination was confirmed by the presence of a vaginal plug (checked for a daily basis). Once observed, the female was removed from the cage containing the male and second female, and housed individually thereafter. Embryos from each litter derived from a pregnant female were randomly and consecutively numbered. Thus each embryo was designated by the male they were sired; by the female from which they were derived (R or L); and their number within the litter. For example, embryo 23L5 was the fifth embryo in the litter derived from dam L who was mated with sire 23. This nomenclature is used in all references and analyses.

3.2.1. Embryonic tissue harvesting

Offspring were harvested at embryonic day 16.5 (E16.5). Dams were humanely euthanised by neck dislocation, and their uteri excised through an abdominal incision. Whole uteri were placed in PBS chilled on ice. Embryos were individually removed from the uterus and whole embryo weight was recorded. Embryos were decapitated with sharp scissors to ensure the cessation of embryonic life. From each embryo, the whole placenta, brain, liver, and tail were harvested. Organs were weighed immediately following dissection, snap frozen in liquid nitrogen, and stored at -80°C.

3.2.2. DNA and RNA extraction

DNA and RNA from frozen E16.5 tissues (liver, brain and placenta) were extracted using the AllPrep DNA/RNA Mini Kit (QIAGEN). Tissues were initially homogenised in 2mL screw cap microfuge tubes containing either 800µl (livers and brains) or 900µl (placentae) of buffer RTL (containing β-mercaptoethanol, as per manufacture's specifications) and Lysing Matrix D beads (MP Biomedicals). Samples were homogenised in a MagNA Lyser (Roche Applied Science, Basel, Switzerland) at 6,500rpm for 60 sec.

Homogenised samples were then spun down at 14,000 rpm for 3 min. For each liver and brain sample, 400µl of the homegenate supernatant was transferred to the DNA spin column, while only 300µl was transferred for placental samples. This ensured that the spin columns were not over-loaded with nucleic acid material. The protocol was followed according to the manufacturer's specifications. DNA was eluted in 100µl of elution buffer, and RNA samples eluted in 40µl of nuclease-free water and stored at -20°C and -80°C,

respectively. DNA yields were approximately 500ng/µl, will RNA yields approximately 2,000ng/µl.

3.2.3. Sex determination

DNA from frozen tail samples was crudely extracted by incubating tissues overnight in a detergent/proteinase lysis buffer (Appendix F). Tail DNA was used to determination embryonic sex using a PCR-based method. This method makes use of two separate PCRs to amplify regions on the mouse sex chromosomes. *Zfy* is a gene found only on the Y chromosome, while *SMCX/Y* is in a region homologous to both the X and Y chromosomes. However, this region on the Y chromosome is 20bp shorter than on the X. Thus, the *Zfy* PCR will generate an amplicon of 200bp in male mice only. The *SMCX/Y* PCR generates a single, intense amplicon band of around 320bp in females, but in males will generate two bands close together of around 320bp and 301bp (corresponding to the X and the Y chromosome amplified regions, respectively). PCR conditions are identical for both regions (Table 12).

Table 12: Sex determination PCR conditions

Reagent	Volume	Cycle conditions		
Sigma PCR Mix	12.5µl	95˚C	5 min	
DNA*	1µl	95˚C	30 sec	40 cycles
Primer F	1µl	60˚C	30 sec	
Primer R	1µl	72˚C	45 sec	
ddH2O	9.5µl	72˚C	10 min	
	25µl			

*obtained directly from lysed tail solution

3.2.4. RNA whole genome expression array of E16.5 tissues

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. The Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, California, USA) was used to determine RNA quality obtained from E16.5 tissues (liver, brain and placenta). The Agilent 2100 bioanalyzer extracts information from RNA electrophoretic traces and allocates a RNA Integrity Number (RIN). The RIN software algorithm allows for the classification of riboeukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact (Agilent, 2004). Only samples with RIN values of ≥8 were used for further analyses in this study.

RNA samples (with a RIN value of ≥8) were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA samples were pooled according to sex and tissue type. Each pool consisted of a total of 250ng of RNA. RNA was obtained in equal proportions from six embryos randomly selected from six independent litters, representing six independent sires from each treatment group. Thus, for each embryo treatment group (embryos of ethanol-treated sires, and sucrose-treated sires) an independent male pool for each tissue and an independent female pool for each tissue were obtained. This gave a total of 12 pools for the RNA array experiment (three tissue types, two genders, two treatment groups). A second biological replicate was created using a further six embryos randomly selected from six independent litters, representing six independent sires from each treatment group. This gave a total of 24 RNA pools, which were used for genome-wide expression analysis.

A whole genome expression array was carried out using the MouseWG-6 v2.0 Expression BeadChip (Illumina, Inc., San Diego, California, USA) to assess differences in expression levels in the tissues of embryos derived from sucrose-treated and ethanol-treated fathers. The

multi-sample format of the BeadChip allows more than 45 200 transcripts and six samples to be simultaneously interrogated. The content of the BeadChip content is sourced from the National Centre for Biotechnology Information Reference Sequence (NCBI RefSeq) database (Build 36, Release 22), is supplemented with probes from the Mouse Exonic Evidence Based Oligonucliotide (MEEBO) and exemplar protein-coding sequences derived from the RIKEN FANTOM2 database (illumina, 2013).

The microarray data pipeline analysis of the whole genome expression data is summarised in Figure 35. Briefly, raw image and data processing was performed using the Illumina BeadStudio Data Analysis Software (Gene Expression (GX) Module). Genes (transcripts) not expressed (not detected on the BeadChip) were removed using the R 'subset' function (Dunn and Smyth, 2008). Between array normalisation was conducted using the lumi v2.8.0 Bioconductor package (Du et al., 2008 24(13):1547-8). A further principal components analysis was conducted to visualise the clustering of RNA expression pools. The Limma v3.12.3 package (Smyth et al., 2002) was used to analyse gene expression data, and identify differentially expressed genes (gene transcripts in EtOH pools whose expression differs from that of Sucrose control pools). Following the identification of differentially expressed genes, Gene Ontology analysis was conducted in DAVID to identify gene enrichment. Further in-depth pathway analysis was conducted using DAVID, Ingenuity Pathway Analysis (IPA) and Cytoscape.

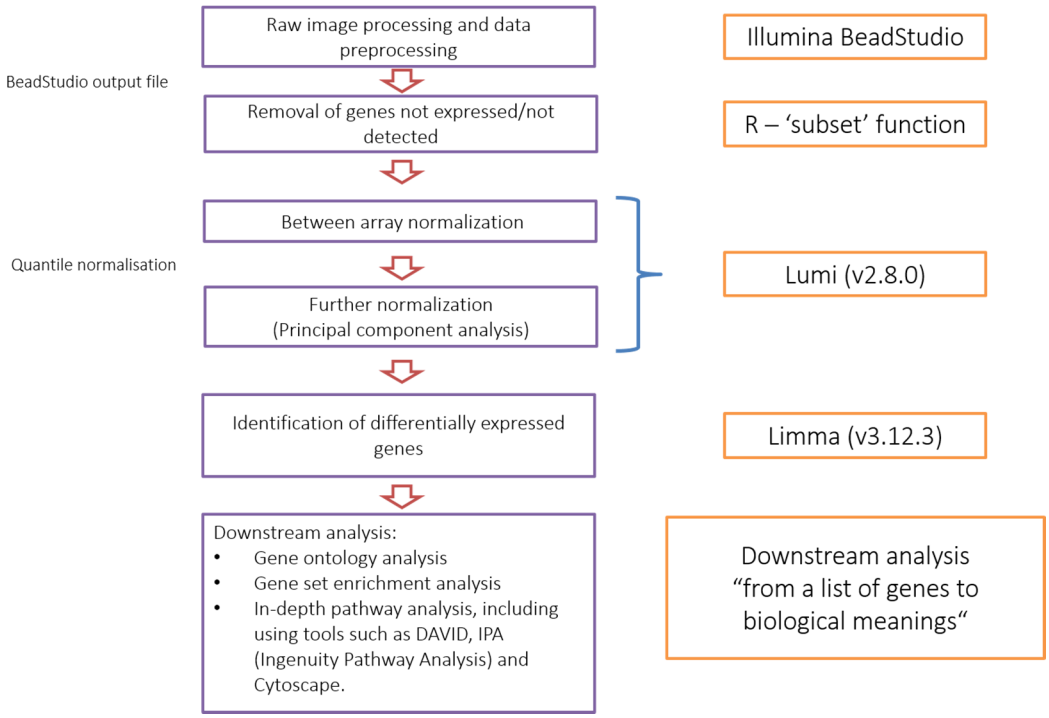

Figure 35: Summary of microarray analysis pipeline of whole genome expression data.

3.2.5. Statistical rationale of embryo organ weight

E16.5 organ weight data (when stratified by treatment group) was initially tested for outliers using the Grubbs' (or extreme studentized deviate) test. Identified outliers were excluded from further analyses. Subsequently, all weight data were tested for normality using the Shapiro-Wilk normality test. Non-normally distributed data was analysed using the non-parametric ANOVA test, Kruskal-Wallis, followed by the Dunn's multivariate comparison posthoc test.

3.3. Results

A total of 192 embryos were obtained from 24 females, representative of 20 sires (Figure 36).

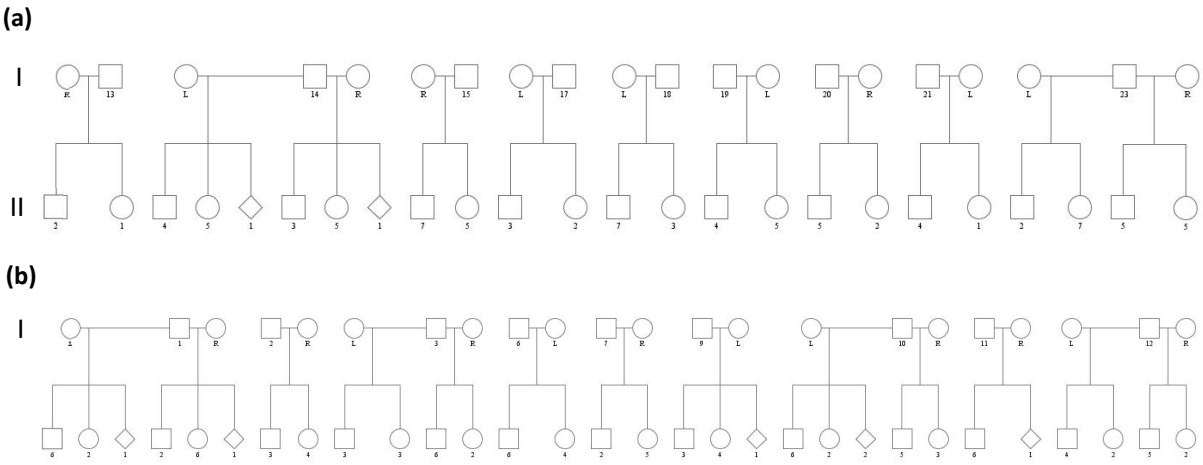

Figure 36: Mouse model pedigree. Males are denoted by □; females by ○; and unknown sex by ◊. Generation I is the parental generation of generation II. Generation I is comprised of treated sires (with identification numbers beside each □) and the non-treated dams (designated as either R or L) that they were mated with. Generation II is comprised of 16.5 day embryos generated from the parental generation I. Numbers below each sex symbol in generation II denote the total number of each sex per litter (a) Ethanol-treated sire pedigree (b) Sucrose-treated sire pedigree.

Although gestational age was calculated from the time at which a vaginal plug was found in each dam (E0.5), this can often be inaccurate and lead to inaccuracies in establishing embryonic age. As mouse embryos undergo a dramatic change in size during this period, it is vital to accurately establish embryonic age for comparative phenotypic analyses between litters. In an attempt to identify litters that were of an inappropriate age (younger or older that E16.5), embryos were assessed morphologically. In addition, whole embryo weights were averaged for each litter to determine a mean litter weight (Figure 37). Evidence

suggests that average E16.5 embryo weight is approximately 600mg (500mg to 700mg), while that of E15.5 is 350mg and E17.5 is 900mg (Eggenschwiler et al., 1997). This range, in addition to morphological analysis, was used to identify litters of inappropriate age. As age difference around E16.5 can have marked differences on organ size and therefore weight, these litters were excluded from analyses (excluding sex ratio and litter size analyses). Four litters (2R, 12L, 13R, and 19L) fell out of this range and were excluded from further analyses. Litter 10R fell marginally out of this range (mean litter weight of 706.8mg) but was included in analyses as it was decided that it was closer to the E16.5 average weight than that of E17.5, and thus most probably E16.5.

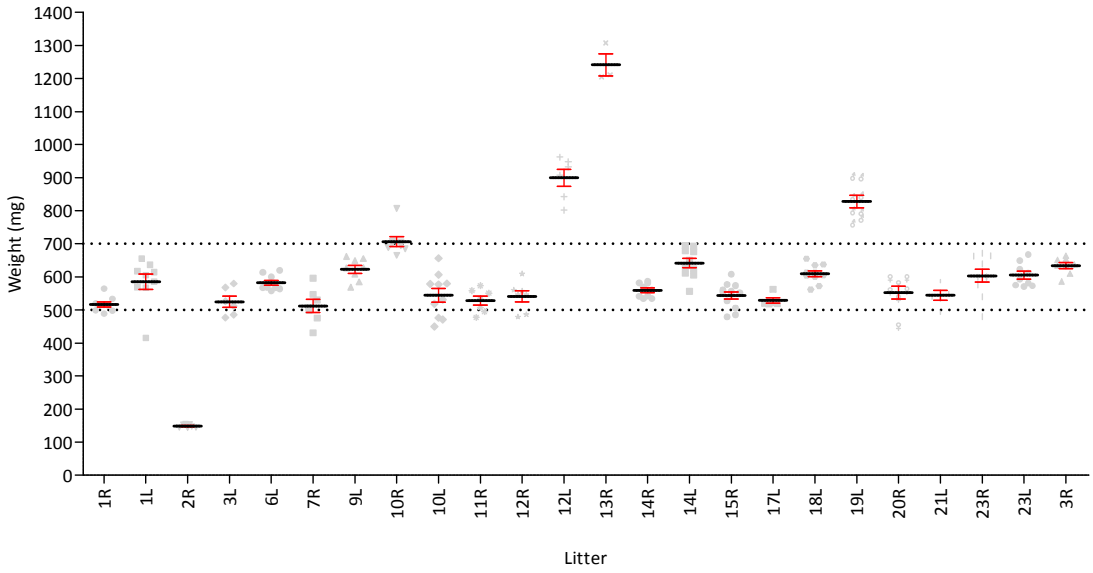

Figure 37: Mean whole embryo weight of E16.5 litters. Average E16.5 litter weights were assessed by averaging the whole embryo weight of all embryos that comprised each litter. An average whole embryo weight range of 500mg to 700mg was used to classify E16.5 litter age (dashed lines).

3.3.1. Descriptive phenotypes of E16.5 embryos

Pregnancy success rate. A total of 11 ethanol-treated males, and 12 sucrose-treated males were mated with two females each. The pregnancy success rate was 50% in both groups (11/22 and 12/24 females successfully impregnated, respectively).

Total embryos. A total of 89 and 102 embryos were sired sucrose-treated and ethanol-treated males, respectively. This is on average 8.09 and 8.50 embryos sired per male in the ethanol-treated and sucrose-treated groups, respectively.

Litter size. The average litter size of the ethanol-treated males was slightly higher (8.09) than the sucrose-treated males (7.85) (Figure 38). There was however a noticeable spread (greater range) of litter sizes noted in the ethanol-sired group.

Sex ratio. Sex was determined using a PCR based system using DNA obtain from embryonic tails (Figure 39). No significant difference in sex ratio was noted between the two sire treatment groups (Figure 38).

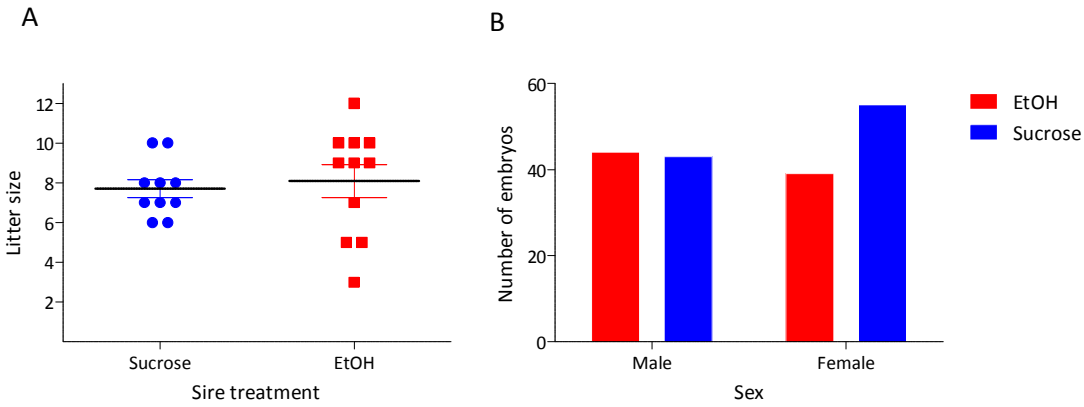

Figure 38: Distribution of litter sizes and sex ratio obtained from treated sires. (A) Litter sizes and (B) sex ratios between the two sire treatment groups.

Resorptions. A total of five and nine resorptions were noted in the ethanol-sired and sucrose-sired embryo groups, respectively. This was on average 0.45 and 0.75 resorptions per litter in the ethanol-sired and sucrose-sired embryos groups, respectively. Although there appears to be a greater occurrence of resorptions in the sucrose-sired group, it must be noted that female 21L, who was mated with an ethanol-treated male, had what appeared to be a pool of resorbed embryos in one of her uterine horns. This was estimated to account for at least three resorbed embryos. Thus, the total number and average number of resorptions per litter for each treatment group are likely to be similar (approximately 0.72 resorptions per litter).

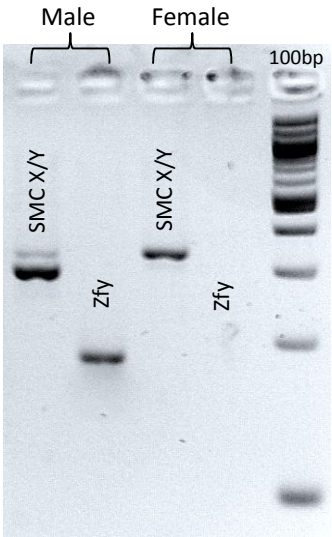

Figure 39: Sex determination agarose gel picture. The PCR based system for determining embryo sex from DNA obtained from E16.5 tails results in two SMC X/Y bands (301bp and 320bp) and a single Zfy band for males (200bp), while females produce only a single SMC X/Y band (320bp).

3.3.2. The effect of paternal alcohol exposure on E16.5 weight

Of the 192 embryos obtained, 19 were excluded from inappropriately aged litters (2R, 12L, 13R, and 19L) and nine due to difficulties in determining sex. Thus, weight analyses were conducted on a total of 164 embryos and their respective organs (Table 13).

Table 13: Statistical analysis of whole embryonic weights and tissue weights of embryos sired by ethanol exposed and sucrose exposed males.

	Sucrose		EtOH		*p*-value
	n	Mean ± Std Dev	n	Mean ± Std Dev	
Litter size	12	7.75 ± 1.36	11	8.10 ± 2.74	0.4924
Male					
Whole Embryo (mg)	54	612 ± 114	40	586 ± 59	0.4305
Placenta (mg)	53	99 ± 19	40	97 ± 26	0.3357
Brain (mg)	53	52 ± 12	40	47 ± 4	0.0213*
Liver (mg)	54	40 ± 7	40	36 ± 5	0.0233*
Female					
Whole Embryo (mg)	35	578 ± 96	35	577 ± 46	0.3557
Placenta (mg)	35	95 ± 23	35	91 ± 21	0.6405
Brain (mg)	35	48 ± 6	35	48 ± 4	0.2924
Liver (mg)	35	39 ± 6	35	37 ± 5	0.5055

* $p \leq 0.05$;

It was observed that there was no significant difference in whole embryo weight and litter sizes between embryos sired by ethanol exposed males and sucrose exposed males. Similarly, placental weight did not differ between the two groups. While not significant in female embryos, male embryos sired by ethanol exposed males showed significant

decreases in brain and liver weight (p=0.0213 and p=0.0233, respectively) when compared to embryos of sucrose exposed males.

3.3.3. Gene expression profiles of E16.5 embryos sired by ethanol-treated males

A total of 24 RNA pools – representative of three tissue types (brain, liver placenta), both sexes (male and female embryos), two treatment groups (EtOH and Sucrose), and two biological replicates – were used for whole genome expression profiling of E16.5 embryos. These were designated as shown in Table 14.

Table 14: Designation of RNA pools used for whole genome expression analysis.

RNA Pool	Embryo sire treatment	Sex	Tissue
1	EtOH	Male	Liver
2	EtOH	Female	Liver
3	EtOH	Male	Brain
4	Sucrose	Female	Liver
5	Sucrose	Male	Liver
6	Sucrose	Female	Brain
7	EtOH	Male	Brain
8	EtOH	Female	Placenta
9	EtOH	Male	Placenta
10	Sucrose	Female	Brain
11	Sucrose	Male	Placenta
12	Sucrose	Female	Placenta
13	EtOH	Male	Liver
14	EtOH	Female	Liver
15	EtOH	Male	Brain
16	Sucrose	Female	Liver
17	Sucrose	Male	Liver
18	Sucrose	Female	Brain
19	EtOH	Male	Brain
20	EtOH	Female	Placenta

21	EtOH	Male	Placenta
22	Sucrose	Female	Brain
23	Sucrose	Male	Placenta
24	Sucrose	Female	Placenta

In the circumstance where a gene was represented by multiple distinct oligonucleotide probes, the probe with highest average expression across all samples was selected for use in further analyses. Thus, a total of 18,592; 16,292; and 17,067 probes (unique gene transcripts) were found to be expressed in the brain, liver and placenta, respectively (following removal of non-expressed genes).

The principal components analysis conducted using lumi revealed that the Sucrose Female Brain Pool clustered with the placental pools (Figure 40). This pool was subsequently removed from further data analyses.

For the ease of subsequent analyses according to tissue type, samples were recoded (Figure 41). Microarray data was normalised (between array normalisation) for all three tissue types (Figure 42) which was used for subsequent analyses.

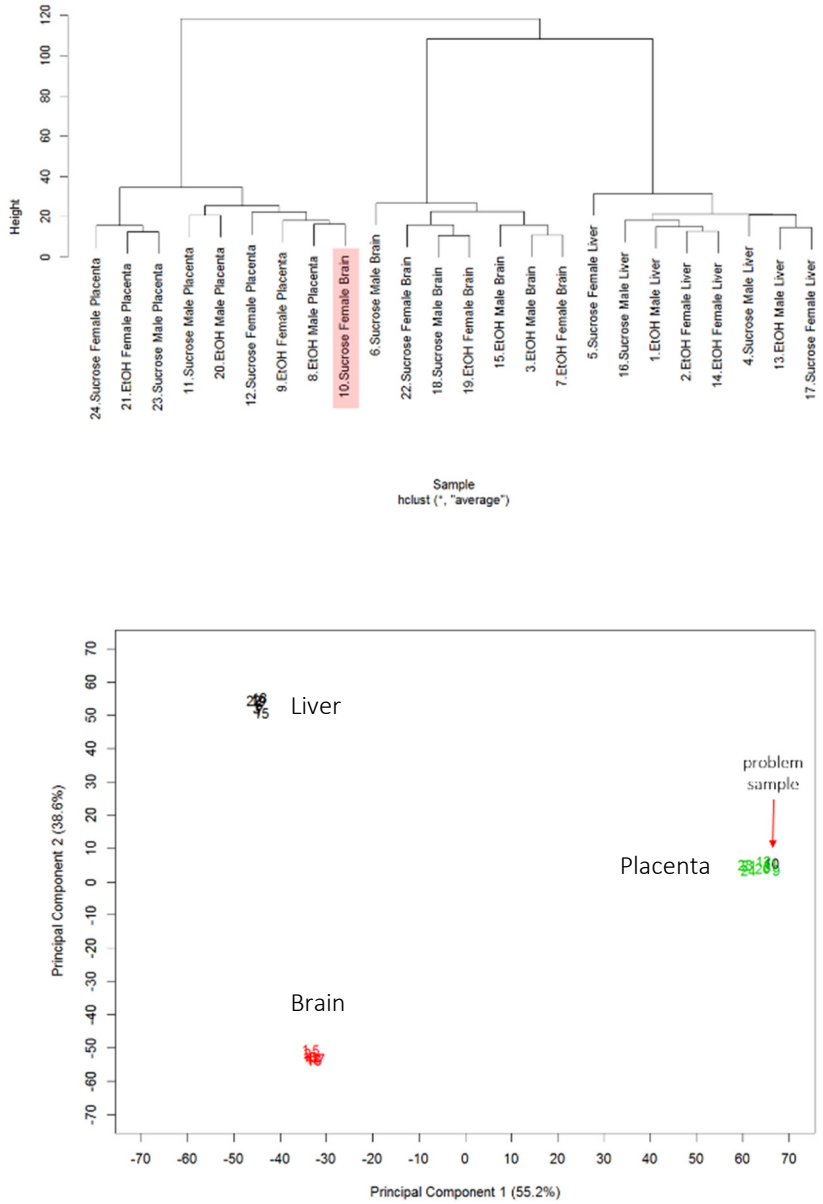

Figure 40: Cluster analysis and PCA plot of embryo gene expression data. The cluster analysis (left) demonstrates discrete clustering of tissue types (brain, liver, placenta), except for Pool 10 Sucrose Female Brain (in red), which clustered with the placental pools. This is also shown in the PCA plot (right).

New ID	Old ID	Samples			
1	3	ETOH Male Brain Rep1	ETOH	Brain	
2	15	ETOH Male Brain Rep2			
3	7	ETOH Female Brain Rep1			
4	19	ETOH Female Brain Rep2			
5	6	Sucrose Male Brain Rep1	CTRL		
6	18	Sucrose Male Brain Rep2			
7	22	Sucrose Female Brain Rep2			
8	1	ETOH Male Liver Rep1	ETOH	Liver	
9	13	ETOH Male Liver Rep2			
10	2	ETOH Female Liver Rep1			
11	14	ETOH Female Liver Rep2			
12	4	Sucrose Male Liver Rep1	CTRL		
13	16	Sucrose Male Liver Rep2			
14	5	Sucrose Female Liver Rep1			
15	17	Sucrose Female Liver Rep2			
16	8	ETOH Male Placenta Rep1	ETOH	Placenta	
17	20	ETOH Male Placenta Rep2			
18	9	ETOH Female Placenta Rep1			
19	21	ETOH Female Placenta Rep2			
20	11	Sucrose Male Placenta Rep1	CTRL		
21	23	Sucrose Male Placenta Rep2			
22	12	Sucrose Female Placenta Rep1			
23	24	Sucrose Female Placenta Rep2			

Figure 41: Recoded RNA pools. RNA pools, consisting of tissues (brain, liver and placenta) obtained from six independent pups from separate litters from six different sires were obtained for whole genome expression arrays for the CAM model. Pools were also stratified according to sex (male and female). The first replicate for the Sucrose Female Brain did not cluster with the other brain pools, and was thus discarded from further analysis. Gene expression data from both male and female pools were combined for gene expression analysis. This resulted in three ETOH and three CTRL (sucrose) datasets for each of the three embryonic tissues (brain, liver, and placenta). For ease of analysis, the original pool IDs were recoded from 1-23.

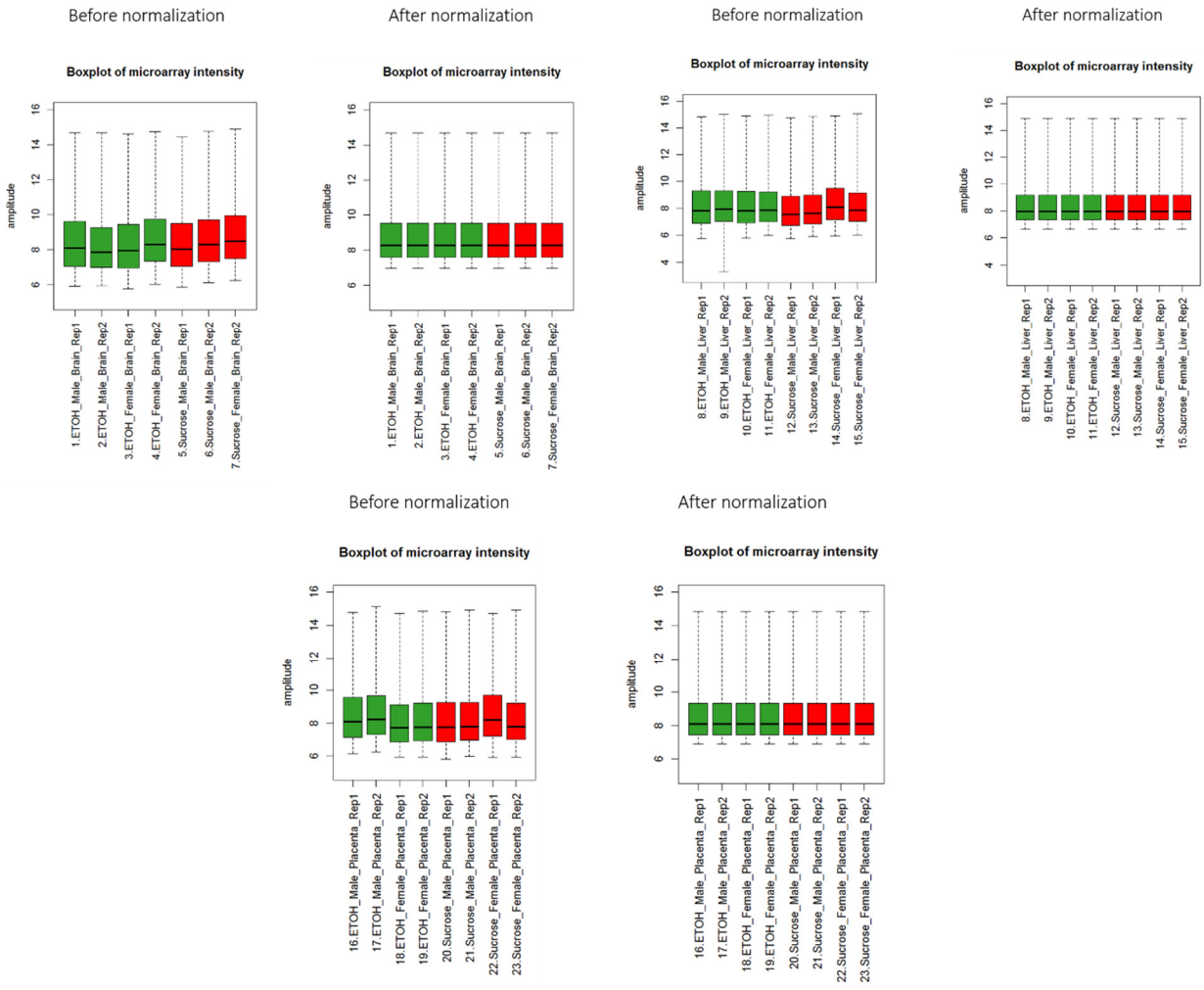

Figure 42: Between-array normalised microarray data of embryo gene expression analysis. Microarray data was normalised across all pools as part of our data QC.

3.3.3.1. Differential gene expression in E16.5 embryos

Volcano plots were used to visualise the 18,592; 16,292; and 17,067 unique gene transcripts that were found to be expressed in the brain, liver and placenta, respectively (Figure 43). For each transcript (gene), Fold Change was plotted against Log Odds. Two *p*-value thresholds were analysed ($p<0.001$ and $p<0.0001$).

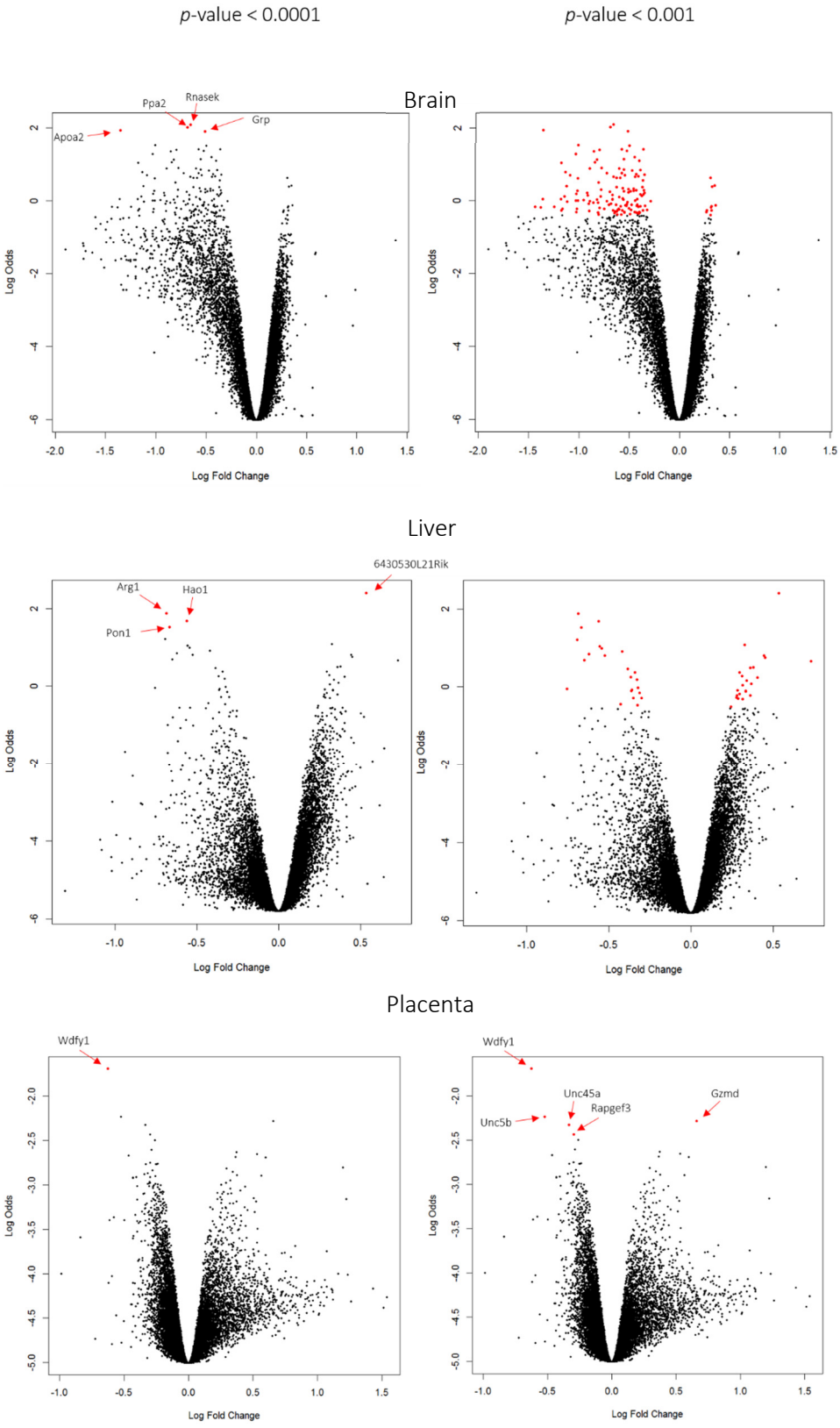

Figure 43: Volcano plots of gene expression data from E16.5 tissues. Unique transcripts (genes) which demonstrated a *p*-value of <0.001 (right) and/or *p*<0.0001 (left) are shown in red.

In the E16.5 brain, it was observed that a total of 145 genes demonstrated significant differential expression in the EtOH group compared to the Sucrose (control) group (10 upregulated, 135 downregulated, $p<0.001$ Appendix E), while only four genes showed a significant differential expression at $p<0.0001$ (all downregulated compared to the control group (*Apoa2*, *Ppa2*, *Rnasek*, *Grp*) (Table 15). Similarly, in the liver, 47 genes showed significant differential expression at $p<0.001$ (24 upregulated, 23 downregulated); while only four genes at the $p<0.0001$ threshold (one upregulated (*643053OL21Rik*), three downregulated (*Arg1*, *Pon1*, *Hao1*). Only five genes demonstrated a significant differential expression at $p<0.001$ (one upregulated (*Gzmd*), four downregulated (*Wdfy1*, *Unc5b*, *Unc45a*, *Rapgef3*) in the placenta, and one gene at $p<0.0001$ (downregulated (*Wdfy1*). The function of these genes is summarised in Table 16.

Table 15: Summary of differentially expressed genes in E16.5 tissues.

	***p*<0.001**	***p*<0.0001**
Brain		
Upregulated genes	10	0
Downregulated genes	135	4
Liver		
Upregulated genes	24	1
Downregulated genes	23	3
Placenta		
Upregulated genes	1	0
Downregulated genes	4	1

Table 16: Summary of function of genes differentially expressed at $p<0.0001$.

Gene	Direction of change	Gene name	Tissue	Fold change	*p*-value	Function
Apoa2	downregulated	*apolipoprotein A-II*	Brain	2.55	6.27E-05	Defects in Apoa2 are the cause of senescence accelerated mouse (SAM), May stabilize HDL (high density lipoprotein) structure by its association with lipids, and affect the HDL metabolism
Ppa2	downregulated	*pyrophosphatase (inorganic) 2*	Brain	1.61	5.65E-05	Member of PPase family
Rnasek	downregulated	*ribonuclease, RNase K*	Brain	1.57	5.16E-05	Endoribonuclease which preferentially cleaves ApU and ApG phosphodiester bonds.
Grp	downregulated	*gastrin releasing peptide*	Brain	1.43	6.48E-05	GRP stimulates gastrin release as well as other gastrointestinal hormones. Operates as a negative feedback regulating fear and established a causal relationship between GRP-receptor gene expression, long-term potentiation, and amygdala-dependent memory for fear.
Arg1	downregulated	*arginase, liver*	Liver	1.61	3.87E-05	Involved in pathway: Nitrogen metabolism; urea cycle
Hao1	downregulated	*hydroxyacid oxidase 1, liver*	Liver	1.48	5.17E-05	Involved in pathway: Organic acid metabolism; glycolic acid degradation
Pon1	downregulated	*paraoxonase 1*	Liver	1.59	6.58E-05	Hydrolyzes a broad spectrum of organophosphate substrates and a number of aromatic carboxylic acid esters. Associated with HDL and ApoA-II downregulated in hyperhomocysteinemia in mouse liver
6430530L21Rik	upregulated	-	Liver	1.45	1.69E-05	
Wdfy1	downregulated	*WD repeat and FYVE domain containing 1*	Placenta	1.54	2.28E-05	This gene encodes a protein which contains a single FYVE domain and multiple WD40 repeats FYVE domain mediates the recruitment of proteins involved in membrane trafficking and cell signalling

3.3.3.2. Gene Ontology analysis of significant differentially expressed genes

A Gene Ontology analysis was conducted on the differentially expressed genes significant at $p<0.001$ using DAVID (Figure 44). According to the GO analysis of embryo brain gene expression, it was demonstrated that there is relevance to alcohol and sucrose treatment. Ontology enrichment terms included "alcohol catabolic process", "glycolysis", "glucose catabolic process", and "carbohydrate catabolic process".

GO analysis of liver gene expression revealed *Pon1* (*paraoxonase 1*) to be highly significantly downregulated (p=6.58E-05) (Table 16). Similar to the brain GO analysis, there was enrichment for glucose metabolism ("polysaccharide catabolic process"). "Response to wounding" was the most significantly enriched GO term.

In terms of placental analyses, only five significantly differentially expressed genes were identified at $p<0.001$. Only a single gene in the placenta showed a significantly differential expression at $p<0.0001$. Based on the relatively small number of significantly differential expressed genes, a Gene Ontology analysis was not possible.

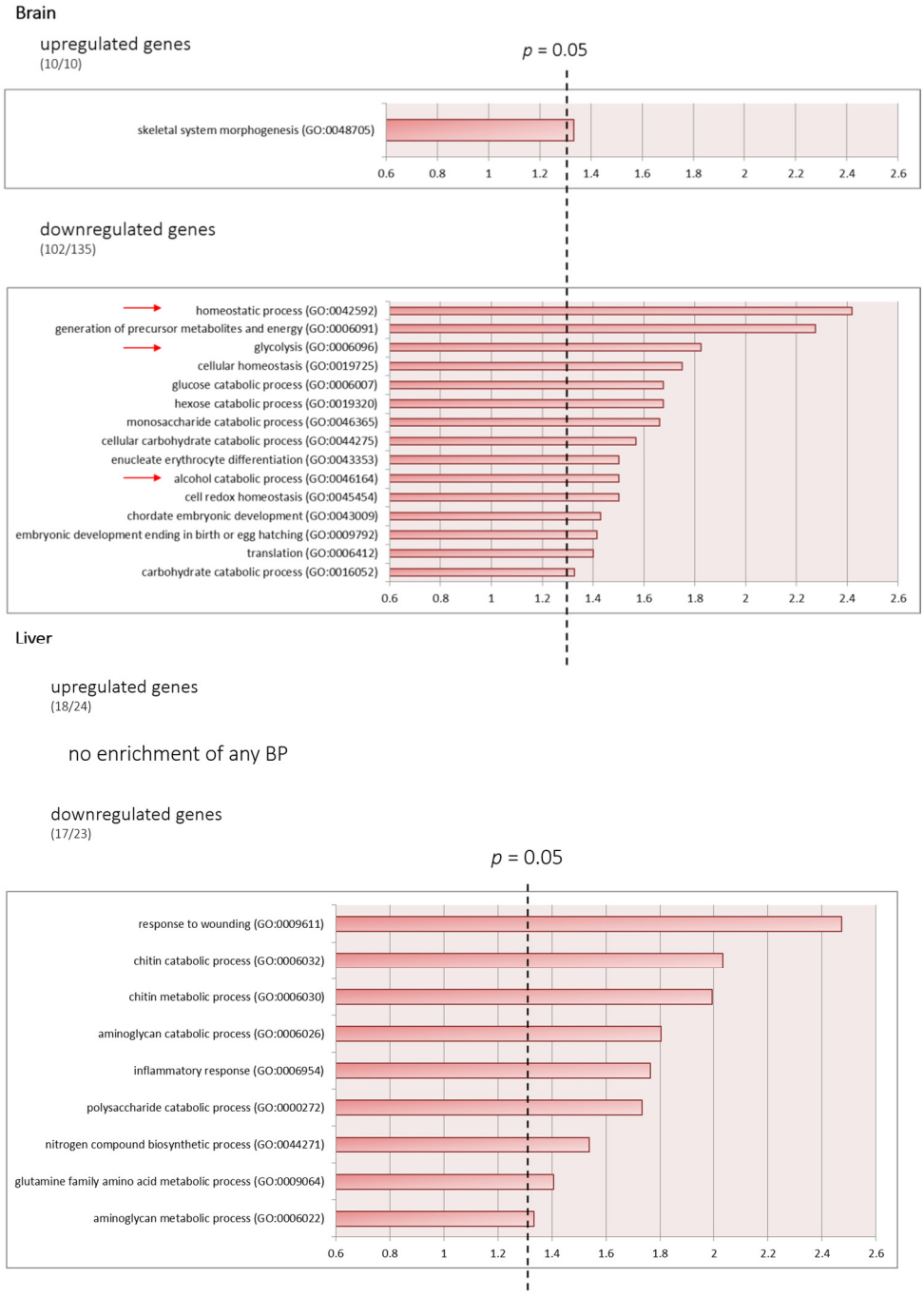

Figure 44: Gene Ontology analysis of genes significantly differentially expressed in the E16.5 brain and liver. A gene ontology analysis was conducted on the genes which demonstrated significant differential expression in embryonic tissues, when compared to controls. Dashed line denotes significance of Gene Ontology terms at p = 0.05. Numbers in brackets denote the number of genes used in the DAVID Gene Ontology analysis / the number of genes identified to be significantly differentially expressed.

3.4. Discussion

In our current study, we hypothesised that chronic paternal alcohol exposure of male mice prior to conception, would induce epimutations in the sperm of alcohol exposed males, which would be transmitted to sired offspring and consequently impact on gene expression and embryonic development. We assessed the weights of embryos sired by ethanol treated males, and compared this, along with brain, liver and placental weights to those of embryos sired by sucrose exposed males. Further to this, we quantified global gene expression in these tissues, in order to determine whether chronic alcohol exposure prior to conception elicits a change in gene expression, and impact on the embryonic phenotype.

3.4.1. Chronic preconception paternal alcohol exposure affects embryo brain and liver weight in male offspring

The embryonic phenotype data obtained in this study used whole embryo and organ weight as a proxy for overall growth as a phenotype at embryonic day 16.5 (E16.5). Our data analysis of embryo phenotype revealed no difference in whole embryo weight, placental weight or litter size between the two paternal treatment groups (ethanol v. sucrose).

While this study did not observe a significant difference in brain or liver weight in female embryos between the two treatment groups, a significant reduction in brain and liver weight was observed in the embryos sired by ethanol treated males (p=0.0213 and p=0.0233, respectively). This finding is contrary to that of Sayal et al (2007) who observed a gender bias in mental health problems of girls exposed to low levels of alcohol in utero. However, Sayal et al also observed evidence of some effect in boys but only at higher cutoffs for severity and with higher levels of drinking. Further to this, animal models of prenatal alcohol exposure

have demonstrated greater vulnerability to stress responsiveness in female offspring (Weinberg, 1992). Other studies have reported that gender bias was not evident in children referred for attention-deficit/hyperactivity disorder as a suspected consequence of prenatal alcohol exposure (Mick et al., 2002).

Although the developing human brain is susceptible to alcohol over all three trimesters of pregnancy, certain regions of the brain or neuronal populations may be more sensitive to alcohol exposure during specific periods of brain development (Coles, 1994; Goodlett and Johnson, 1997; Maier et al., 1996). The third trimester of human embryonic development coincides with the neonatal period of brain development in rats (Bayer et al., 1993; Dobbing and Sands, 1973, 1979; West, 1987). This is a particularly sensitive period, where alcohol exposure has been shown to induce cell death and permanent loss of neurons in the cerebellum, hippocampus, and certain cortical and subcortical neuronal populations (Bonthius et al., 1992, 2001; Bonthius and West, 1991; Goodlett and Johnson, 1999; Goodlett and Lundahl, 1996; Goodlett et al., 1998; Ikonomidou et al., 2000; Pauli et al., 1995a). Bonthius and West (1991) observed that neonatal rats exposed to 4.5-6.6g/kg/day of ethanol showed significant decreased in neonatal brain weight (total brain weight, forebrain and cerebellum). Johnson and Goodlett (2002) further observed that exposure of neonatal rats to 5.25g/kg/day of ethanol during the latter part of the brain growth spurt period (between postnatal day 7 and 9) caused enduring deficits in spatial learning, but only in males. Two other reports (Girard et al., 2000, 2001) found that neonatal binge alcohol exposure resulted in deficits on a spatial working memory version of the Morris water maze in adult male rats. Similarly, Zimmerberg and Scalzi (1989) observed that rats prenatally exposed to ethanol resulted in behavioural dysfunctions that persisted into adulthood. While both sexes appeared to have impairment of neural areas associated with reference memory, male rats appeared to be more vulnerable in neural regions associated with working

memory. These latter studies provide evidence for increased vulnerability of the male brain to prenatal alcohol exposure, which is supported by our current study, where reduced brain weight was only evident in male offspring.

Together, these findings demonstrate that prenatal alcohol exposure (including the neonatal period in rats, which is equivalent to the third trimester in humans) can result in reduced brain weight in offspring, which has been associated with male gender bias. Our findings show evidence of a similar reduced male brain weight phenotype in embryos whose sires were chronically exposed to ethanol. That is, indirect parental (paternal) alcohol exposure can elicit phenotypic defects in offspring that are equivalent to that seen in offspring directly exposed to ethanol in utero (prenatally via maternal alcohol exposure). Furthermore, reduced brain weight is a hallmark feature of foetal alcohol syndrome in humans.

While exposure of adult rats to alcohol appears to result in increased liver weight (in both males and females) (Tadic et al., 2002), prenatal exposure to alcohol is generally associated with decreased embryonic liver weight (Henderson et al., 1979; Sanchis and Guerri, 1986). Liver abnormalities have also been observed in patients with foetal alcohol syndrome. Habbick et al (1979) noted thick, sclerotic central veins along with features typical of congenital hepatic fibrosis and cystic disease of the kidneys. These latter finding are synonymous to that of the current study, where we observed a reduction in (male) embryonic liver weight associated with chronic prenatal paternal alcohol exposure (p=0.0233).

Overall, it appears that although offspring were not directly exposed to alcohol, but rather indirectly via exposed male gametes, at least male offspring (embryos) exhibited a growth-restricted phenotype of the brain and liver that are known to be affected in foetal alcohol syndrome.

3.4.2. Chronic preconception paternal alcohol exposure dysregulates gene expression in embryos

Both human and animal studies have been used to investigate whether vulnerability to stress, as an environmental agent, is epigenetically heritable and transmissible through the germline. Corticotrophin releasing factor type 1 (CRF1) is an essential component in stress response. Zaidan et al. (2013) assessed *CRF1* expression levels in the brains and ova of stressed female mice and in their respective offspring. CRF1 mRNA expression levels were found to be elevated in the frontal cortex of mature female mice under chronic stress, which was also observed in the brains of their neonatal offspring. Bromer et al (2010) also demonstrated that Bisphenol A (BPA) induced changes in the homeobox gene *Hoxa10* (which controls uterine organogenesis) in the reproductive tract of mice exposed to BPA in utero. These changes were associated with a concomitant >50% decrease in DNA methylation at the *Hoxa10* promoter and intron.

Alcohol has also been shown to affect gene expression. As discussed previously, in vitro alcohol exposure of NSCs decreased DNMT1 expression. Lee et al (2004) observed that in utero alcohol exposure of C57BL/6 mice altered gene profiles, including *palate, lung and nasal epithelium clone* (*plunc*), which was found to be highly expressed in the craniofacial region, specifically in upper airways and nasopharyngeal epithelium. Similar observations were noted by Hard et al (2005), who observed ethanol-induced downregulation of several genes in the brain of prenatally exposed mice. Wang et al (2009) investigated the effect of alcohol exposure on the expression of 509 micro RNAs in developing mouse embryos in culture. These authors observed that ethanol exposure resulted in foetal teratogenesis, mental retardation and impaired locomotor activity, synonymous with a FAS phenotype.

They further observed that several miRNAs that were both aberrantly upregulated and downregulated in the foetal brain. In a later and more comprehensive study, Kleiber et al (2012) demonstrated long-term alteration to the brain transcriptome in an in utero exposure mouse model of FASD. Pathways affected included those involved in apoptosis, cellular differentiation and maturation, and epigenetic programming.

From the findings above, investigations into whether paternal environmental factors could alter gene expression in their offspring were initiated. In recent years, there has been emerging evidence on how environmental exposures induce epigenetic changes in the paternal germline that consequently affects subsequent generations. Vassoler et al. (2013) reported that male offspring sired by cocaine-treated males exhibit increased expression levels of cortical *brain-derived neurotrophic factor* (*Bdnf*), which confers a cocaine resistant phenotype. Carone et al (2010) demonstrated that the offspring of male mice exposed to a low-protein diet displayed an increase in expression of hepatic genes involved in lipid and cholesterol biosynthesis and reduced levels of cholesterol esters compared to offspring sired by males that were fed a control diet. In addition, reduced representation bisulfite sequencing (RRBS) revealed that there were modest (~20%) alterations in cytosine methylation in the livers of offspring depending on paternal diet, as well as reproducible changes in methylation over a potential enhancer for the key lipid regulator peroxisome proliferator-activated receptor alpha (PPARα).

Taken together, this evidence suggests that paternal environmental exposures are able to dysregulate gene expression in their offspring. It is thus plausible that excessive alcohol intake by males prior to conception may affect gene expression in sired offspring (possibly via an epigenetic mechanism).

3.4.3. Biological processes identified by Gene Ontology analysis of differential gene expression in embryo brains

3.4.3.1. Skeletal system morphogenesis is associated with FAS

The only significant (p<0.05) biological process identified in the embryonic brain using DAVID Gene Ontology analysis was *skeletal system morphogenesis*. According to the Jackson Lab, skeletal system morphogenesis is defined as "the process in which the anatomical structures of the skeleton are generated and organised".

In utero exposure to alcohol can occur anywhere between conception and just prior to birth, and for varying periods of time. Alcohol exposure during different periods of brain development results in regional differences in cell loss depending on the period of brain development at which the insult occurs, and illustrates the variability of alcohol-induced neuronal loss (Maier et al., 1996). Both Goodlett and Lundahl (1996) and Thomas et al (1996) further observed that exposure of rats to alcohol during postnatal 4-5 (third trimester equivalent in humans, during the phase of initial dendritic outgrowth of the Purkinje cells) was associated with greater Purkinje cell loss and produced significantly more severe motor deficits and significantly more severe reductions in cerebellar and brainstem weights than did exposure on postnatal 8-9. These studies demonstrate that early stage in utero exposure is generally associated with more severe outcomes on brain development than that of latter stage exposure. This is likely due to the simple fact that the foetus is being exposed for a greater period of time during brain development and more damage (cytotoxic and/or epigenetic) is inflicted. However, this might also allude to the fact that in early stage

exposure, the progenitor cells are affected, and subsequently give rise to aberrant developmental pathways and impaired organogenesis. In our model, abnormal sperm epigenetic patterns that maybe be inherited by offspring, are essentially the founding genetic material for progenitor cells of the embryo and organogenesis systems, including that of the skeletal system.

At the outset, the fact that a biological process for skeletal development is enriched in the brain appears misplaced. However this might imply that either pathways that regulate bone development also regulate brain development; or that signals from the brain regulate skeletal development in the body.

Synonymous pathways

It is known that bone morphogenetic proteins (BMPs) regulate embryonic skeletal development (Ducy and Karsenty, 2000; Hogan et al., 1996). However, it has been widely noted that several *Bmp* genes are expressed in localised patterns during embryonic mouse brain development (Jones et al., 1991; King et al., 1994; Lyons et al., 1995). BMPs have been shown to be crucially involved in the development of the both the central and peripheral nervous system (brain and sympathetic neurons) (Liu and Niswander, 2005; Urist, 1997). BMPs also promote neural crest cell migration, mediate neural crest apoptosis in the hindbrain and direct neural crest differentiation into sympathetic neurons (Liu and Niswander, 2005). In Drosophila, BMPs have been found to regulate dorsal forebrain development (Furuta et al., 1997). This study also found that BMP proteins locally inhibit cell proliferation and increase apoptosis in telencephalic neuroectoderm explants of mouse embryos. These results provide evidence that BMPs function during regional morphogenesis of the dorsal telencephalon by regulating specific gene expression, cell proliferation and local cell death. The role of BMPs in forebrain and cerebellum development have also been

observed in the mouse brain. BMP4 protein enrichment of mouse forebrain explants resulted in a decrease in cell proliferation and an increase in cell death (Liu and Niswander, 2005). The current understanding of BMP involvement in neural development is that BMP signalling is necessary for the development of the most dorsal structure of the forebrain whereas the development of the ventral forebrain is dependent on the inhibition of BMP signalling. It is further interesting to note that the *Bmp4-Bmpr1a* genetic pathway that functions in lip fusion, and reveal that *Bmp* signalling has distinct roles in lip and palate fusion (Liu et al., 2005). Cleft lip and palate has also been associated with foetal alcohol syndrome. Bierich et al (1975) reported that 39% of FAS children have highly arched palates, whereas 7% have cleft palates. Abnormal arch heights have also been observed in mouse models of FAS (Sulik and Johnston, 1983) and cleft palates (submucosal and bilateral) have been noted in infants exposed to alcohol in utero and FAS patients (DeRoo et al., 2008; Swayze et al., 1997).

Skeletal dysmorphogenesis is associated with FAS

FAS is characterised by craniofacial abnormalities. A close interconnection exists between the development of the face, the craniofacial skeleton, and the brain (Kjaer, 1995).

The key mechanism behind the typical facial features and CNS abnormalities that develop simultaneously in FAS, is cellular death at the rostral boundary of the preclosure forebrain and the corresponding cell population involved in the postclosure telencephalic midline. Evidence suggests that a critical pathogenic feature associated with ethanol induced malformations is cell death (Dunty et al., 2001). Programmed cell death (PCD) is a genetically controlled and highly regulated pathway that occurs in every developing embryo, and deviation from this pattern may lead to dysmorphogenesis (Dunty et al., 2001). Apoptosis is a form of PCD and accounts for most cell death (as opposed to necrosis which is the death of

a cell or tissue caused by external factors. such as trauma or infection). Apoptotic neurodegeneration is triggered by ethanol through a twofold mechanism: blockade of the N-methyl D-aspartate (NDMA) glutamate receptors and excessive activation of gamma-aminobutyric acid (GABA) receptors (Dunty et al., 2001). There are multiple cell lines that are sensitive to ethanol-induced cell death. These populations include: the epithelium that lines the nasal cavities, the neural crest, epibranchial placodes and subpopulations of the optic placodes or vesicles (Dunty et al., 2001). Placodes are specialised thickenings in the surface ectoderm which focally regulate skeletal and dental development within the underlying mesenchyme.

Dlx genes are all expressed in spatially and temporally restricted patterns in craniofacial primordia, basal telencephalon and diencephalon, and in distal regions of extending appendages including the limb and the genital tubercle (Acampora et al., 1999). *Dlx5* is expressed much earlier than other *Dlx* genes during development in territories that define the rostral and lateral border of the neural plate when these regions have organizing activities that pattern the adjacent rostral prosencephalon. Additionally, *Dlx5* and *Dlx6* are expressed in all developing bones from the time of initial cartilage formation onward. *Dlx5* has been shown to be important in the control of bone differentiation. While *Dlx5-/-* mice are not viable, *Dlx5+/-* mice present with a complex phenotype characterised by craniofacial abnormalities (skull alterations and defects in the head cartilage skeleton), severe malformations and delayed ossification of the roof of the skull (Acampora et al., 1999). The role of *Dlx* genes as critical regulators of mammalian limb development were later confirmed by Robledo et al (2002), who observed severe craniofacial, axial, and appendicular skeletal abnormalities in *Dlx5* and *Dlx6* knockout mice. It has been further observed that ectodermal expression of *Dlx5* is required for the development of olfactory and optic placode-derived epithelia (Depew et al., 1999). In our current study, we observed that *Dlx2* was

downregulated in the brains of embryos sired by ethanol exposed males, which was associated with a reduction in brain weight of male embryos.

The facial changes of foetal alcohol syndrome (FAS) closely resemble those of a mild form of holoprosencephaly (Siebert et al., 1991), which is characterised by a mid-line anterior neural plate deficiency which leads to olfactory placodes being positioned too close to the mid-line, which commonly causes cleft lip and palate. The principal craniofacial features of FAS are reduced or missing mid-line components of the brain. Deficient development of the mid-line region of the neural plate results in insufficient bilateral separation of the olfactory placodes, which are derived from the anterior neural plate (Johnston and Bronsky, 1995).

Animal models have also shown evidence of alcohol-induced skeletal abnormalities. Exposure of the developing zebrafish has been associated with neurobehavioral abnormalities, skeletal dysmorphogenesis (Carvan et al., 2004), and abnormal craniofacial skeletal development, which is synonymous with the FAS phenotype (Loucks and Carvan, 2004). Similar observations have been noted in humans afflicted with FAS, where children prenatally exposed to alcohol displayed skeletal malformation of the hands and feet (tetraectrodactyly and camptodactyly) (Herrmann et al., 1980), as well as delayed skeletal maturity which was associated with poor performance on motor tasks (Mattson et al., 2011).

Skeletal malformations associated with paternal preconception alcohol exposure

In a recent study by Lee et al (2013) investigated transgenerational toxicities in offspring, both in foetal and postnatal stages with paternal exposure to various alcohol concentrations. This study observed agenesis and skull malformation (exencephaly) in E16.5 offspring sired by ethanol-exposed males (4g/kg). They further noted that these malformations were associated with significant changes in postnatal offspring organ weight. These authors

concluded that paternal alcohol consumption prior to conception represents a potential risk to foetal and postnatal development. This study shows striking seminaries to our current study, where we observed reduced organ weight in male embryos of alcoholised male mice. However, in contrast to our study where significant weight reduction in male livers was noted, Lee et al (Lee et al., 2013) observed decreased organ weight in the embryonic lung of male offspring. Interestingly, a sex bias was also noted, where weight reduction was only evident in male embryos. Similarly, Lee et al did not observe differences in whole embryos weight in either sex, as was evident in our study.

Our gene ontology analysis revealed that two genes comprised the skeletal morphogenesis biological process in the brain of E16.5 embryos. It was observed that *Gli3* and *Mmp2* were upregulated. Mutations in *Gli3* have been associated with several diseases, including Greig cephalopolysyndactyly syndrome (Hui and Joyner, 1993), Pallister-Hall syndrome (Kang et al., 1997), preaxial polydactyly type IV and postaxial polydactyly types A1 and B (Radhakrishna et al., 1999). Greig cephalopolysyndactyly syndrome is an autosomal disorder that affects limb and craniofacial development (Gollop et al., 1984). This disorder is characterised by one or more extra fingers or toes (polydactyly) and/or the fusion of skin between the fingers and toes (cutaneous syndactyly). This disorder is also characterised by craniofacial defects that include widely spaced eyes (ocular hypertelorism), an abnormally large head size (macrocephaly), and a high, prominent forehead (Hui and Joyner, 1993). Rarely, affected individuals may have more serious medical problems including seizures, developmental delay, and intellectual disability (Biesecker, 2008).

Sonic hedgehog (*Shh*) is a highly conserved foetal morphogen that partakes in the regulation of cellular proliferation, differentiation, and embryonic patterning by activating the *Hedgehog* (*Hh*) signal pathway (Hammerschmidt et al., 1997). *Gli3* is proposed to negatively

regulate *Shh* by restricting its expression and influence to the posterior mesoderm (Litingtung et al., 2002; Sasaki et al., 1999). During embryogenesis, *Shh* is expressed in the floor plate of the neural tube, cardiac mesenchyme, throughout the notochord, and in the posterior portion of the limb buds, where it regulates patterning of the ventral neural tube (Echelard et al., 1993), and the anterior-posterior limb axis (Riddle et al., 1993). More recently, Wilson et al (2012) demonstrated that primary cilia and *Gli3* activity regulate cerebral cortical size. Chick embryos exposed to ethanol has been shown to result in downregulation of *Gli3* and neural crest cell death (Ahlgren et al., 2002), and *Gli3*$^{-/-}$ in mice reduces apoptosis in neural tube (Aoto et al., 2002). Mutations in human *Shh* have been associated with holoprosencephaly, in which the foetal forebrain fails to divide into the bilateral cerebral hemispheres (Wallis and Muenke, 2000). Holoprosencephaly is a severe phenotype of FASD in both humans and mouse models (Coulter et al., 1993; Ronen and Andrews, 1991; Siebert et al., 1991). In support of *Hedgehog*'s involvement in FAS, Lombard et al (2007) identified the Hedgehog signalling pathway to be over-represented among the top candidate genes that these authors identified in a computational selection and prioritisation of candidate genes for FAS. In support of this, Li et al (2007) observed that hedgehog cholesterol modification and signalling is impaired by alcohol exposure in zebrafish embryos exposed to alcohol in culture.

Shh and *Gli3* have been shown to be exclusively expressed in the limb buds of developing mouse embryos, where *Gli3* has been shown to regulate the expression of *Shh* (Buscher et al., 1997) which regulates skeletal patterning and development (Mo et al., 1997). Motoyama et al (2006) have shown that the limbs of mouse *Gli3*$^{-/-}$ embryos manifest with severe polysyndactyly, suggesting that *Gli3* is required not only for restricting the number of digits but also for proper apoptosis in interdigital regions (Bose et al., 2002). This suggest that intact Gli3 is required for physiological apoptosis during normal limb development.

Similar to the role of *Gli3*, *Matrix metalloproteinase 2* (*Mmp2*) has been shown to play a role in skeletal development. Mutations in human MMP2 causes severe skeletal defect syndrome, who display hyperextension of metacarpophalangeal joints, flexion contractures of large joints, dysmorphic faces, and significant growth restrictions (Egeblad et al., 2007; Rouzier et al., 2006). Overexpression and high activity of MMP2 has been associated with metastatic breast cancers and serves as a prognostic indicator of clinical outcome (Aye et al., 2004). In parallel to this finding, it has also been observed that alcohol consumption is associated with advanced and invasive breast tumours (Aye et al., 2004). Using a cell culture model, Aye et al (2004) observed that alcohol exposure enhanced the invasive potential of breast cancer cells, which was associated with MMP2 activation. This finding has been supported by Partridge et al (1999) who observed that long-term alcohol exposure increased Mmp2 enzymatic activity of the rat aorta.

3.4.3.2. Enhanced energy and alcohol metabolism may be a consequence of foetal programming

Enrichment of biological process terms for energy metabolism and alcohol catabolism were evident from our GO analysis of gene expression profiles significantly altered in the brains of E16.5 embryos of alcoholic sires when compared to those of control (sucrose exposed) sires.

Several studies have observed metabolic disturbances of offspring who were born to dams who were exposed to high-fat diets. For example, Borengasser et al (2014) exposed pregnant dams to a high caloric diet during pregnancy ("obese" dams). Subsequently, their offspring were postnatally exposed to high-fat diets. As a consequence, they gained greater body weight and fat mass when compared to controls. These authors observed strong suppression

of genes involved in metabolism (including PPARα) and circadian rhythm in offspring livers. They further noted that alterations in PPARα transcription were associated with epigenetic alterations in H3K4me3 and H3K27me3 histone marks near the PPARα transcription start site. These authors concluded that alterations to the circadian machinery contributed to impaired liver metabolism in response to exposure to a high-fat diet, prior to the development of obesity. That is, there was evidence of metabolic programming in the liver of the offspring exposed in utero to the maternal environment, which poised the offspring to be hyperesponsive to a high-fat diet, promoting the development of obesity later in life. A further example of an adaptive response to conditions related to that of dietary intake can be seen by the regulation of negative energy balance and weight loss. It has been noted that the increase of food intake is associated with a reduction of energy expenditure. This is thought to be induced by mechanisms that initiate energy deprivation or chronic energy restriction that enables the efficient recovery of lost weight, comprising an important protective mechanism for survival (Schwartz et al., 2000). The concept of foetal programming in response to the maternal environment as it applies to the foetal origins of disease hypothesis has been explored further in Chapter 1 (Ozanne and Constancia, 2007).

Based on the above and the concept of foetal programming, it may not have been unexpected to observe an enrichment of energy metabolism biological processes in the current study, if a direct foetal (embryo) exposure model was employed. That is, if an in utero model was employed in our current study, control mice would have been directly exposed to excessive quantities of sugar (sucrose), and the need to metabolise these sugars would likely have elicited an upregulation of genes involved in sugar metabolism. This adaptive phenotype for “advanced sucrose metabolism” may have been programmed in the developing foetus, and hence would have exhibited an altered profile of genes related to energy metabolism. Consequently, when comparing the expression profiles of “control”

embryos to those of ethanol-exposed embryos, an apparent downregulation of genes involved with sugar metabolism would have been observed in the ethanol-exposed mice when compared to controls. However, this finding would perhaps have been more correctly interpreted as an upregulation of these genes in sucrose-exposed mice. However, what is striking from the finding that genes involved in sucrose (energy) metabolism were enriched in our GO analysis, is that offspring were not directly exposed to sucrose (or ethanol).

From the above, this finding might suggest evidence for a paternally-mediated, indirect foetal (epigenetic) programming mechanism, whereby male mice chronically exposed to sucrose, undergo epigenetic reprogramming in response to dietary intake, which includes that of the male germline. This adaptive programming is then subsequently inherited by sired offspring, who exhibit an altered transcriptome that is poised for high sucrose metabolism. In support of paternal foetal programming, human epidemiological studies have revealed both maternal and paternal effects on offspring birth weight and type-2 diabetes (Drake and Walker, 2004). Extensive studies in the Pima Indian community, which has a high prevalence of maternal diabetes, have shown that low birth weight is associated with the subsequent development of type 2 diabetes only if paternal diabetes is also present (Lindsay et al., 2000). Epigenetic modification of genes has been proposed to underpin the association of lower offspring birth weight with paternal diabetes in Pima populations (Lindsay et al., 2002). Further to this, Drake et al (2005) observed that offspring of male rats prenatally exposed to glucocorticoids showed reduced birth weight, glucose intolerance, and elevated hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity.

In our current study, the energy metabolism biological process was enriched in the offspring brain. Neuronal regulation of hepatic glucose metabolism in mammals has been studied extensively (Shimazu, 1987). The brain continuously transduces input from neural, hormonal

and nutrient-related signals. Insulin and leptin are able to elicit signals that convey information to the brain regarding long-term energy stores, while information regarding short-term energy availability is conveyed by nutrient related signals such as glucose and free-fatty acids. In response to this input, the brain makes adjustments to output systems that control food intake, energy expenditure, hepatic insulin sensitivity and glucose uptake into responses that maintain both energy- and glucose-homeostasis (Morton, 2007). Beyond the hypothalamus, functional leptin receptors are expressed in the hippocampus, hindbrain pyriform cortex and other olfactory processing areas. Leptin is mainly produced by adipose tissue and can cross the blood–brain barrier (BBB) to act on the hypothalamus and other central nervous system (CNS) targets to reduce food intake (Kastin and Pan, 2006). There is evidence that leptin participates in the metabolic disturbance associated with chronic alcoholism (Balasubramaniyan and Nalini, 2006). Leptin deficiency has been shown to contribute to the pathogenesis of alcoholic fatty liver disease in mice (Tan et al., 2012), and forced alcohol ingestion has been associated with increased permeation of leptin across the blood brain barrier into the brain (Pan et al., 2008).

There exists an interesting link between glucose tolerance and prenatal alcohol exposure. Early studies demonstrated glucose tolerance and insulin response in offspring of ethanol-treated pregnant rats (Villarroya and Mampel, 1985). More recently, Chen et al (2003) investigated postnatal glucose metabolism in offspring prenatally exposed to alcohol. These authors observed that at 13 weeks of age, body weight and β-cell mass of offspring prenatally exposed to ethanol were normal, but plasma glucose and insulin after a glucose challenge were increased compared with controls. This demonstrated that prenatal alcohol exposure impairs glucose tolerance in the offspring by both inducing insulin resistance and β-cell dysfunction. This has been further supported by Yao et al (2013) who observed glucose intolerance with increased hepatic gluconeogenesis and histone deacetylases in

adult rat offspring prenatally exposed to alcohol. These authors also observed that prenatal ethanol exposure during pregnancy increased gluconeogenesis, gluconeogenic genes (including PEPCK), and oxidative and endoplasmic reticulum stress in adult offspring. Together, these findings may suggest that ethanol exposure is associated with enhanced glucose metabolism. This would support the findings of our GO analysis, where energy metabolism was enriched in the brains of embryos sired by males chronically exposed to alcohol prior to conception.

The above reasoning may also hold true for the enrichment of the alcohol catabolism biological process in the brain of embryos sired by ethanol treated males. Three genes involved with alcohol catabolism clustered within this biological process: *Malate dehydrogenase 1* (*Mdh1*), *Phosphoglycerate kinase* (*Pgk1*) and *Enolase 1* (*Eno1*).

Mdh exists in two protein forms, namely, cytoplasmic (Mdh1) and cytosolic (Mdh2). These malate dehydrogenases catalyse the conversion of oxaloacetate and NADH to malate and NAD+ (Figure 45). The regulation of the NADH/NAD ratio is important during alcohol metabolism, as excess NADH decreases ADH activity and alters the cellular redox state (Meijer et al., 1975).

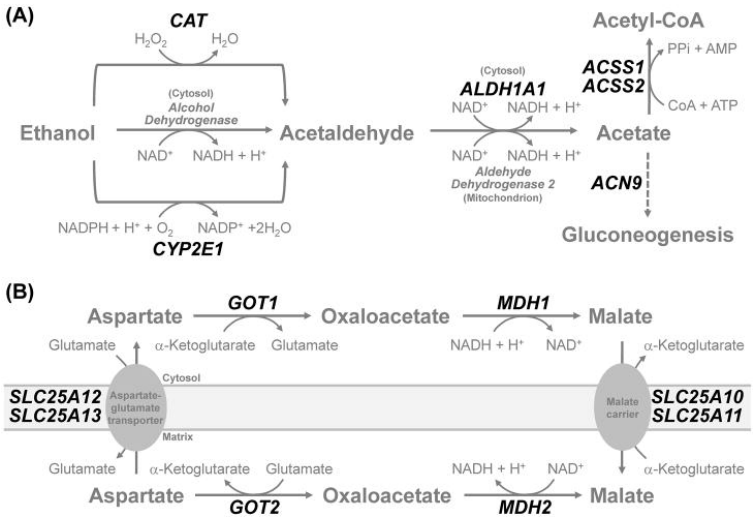

Figure 45: Alcohol (ethanol) metabolism pathways. (A) The metabolism of the carbon skeleton backbone of ethanol. (B) The bidirectional malate-aspartate NADH shuttle. (Lind et al., 2012).

Several studies have investigated the genetic aspects of alcohol (ethanol) tolerance. Previous findings have demonstrated that the hippocampus is involved in the development of alcohol tolerance (Hoffman and Tabakoff, 1989; Ludvig et al., 2001). Further to this, alcohol-preferring (P) rats have been shown to develop acute tolerance to alcohol more rapidly than alcohol-non-preferring (NP) rats (Waller et al., 1983). Edenberg et al (2005) investigated the expression profiles in the hippocampus of P and NP rats in an attempt to gain insight into the genetics of alcohol tolerance. These authors observed 129 genes that were significantly differentially expressed in P rats when compared to NP rats. Among these genes, they noted a 2.18 fold change (FC) increase in *Pgk1* expression ($p<0.00001$), a 1.15 FC decrease in *Mdh1* expression (p=0.00007), and a 1.19 FC decrease in *Eno1* expression in the hippocampus of alcohol preferring rats than that of alcohol non-preferring rats – all of which were involved in metabolism. These findings suggest that *Pgk1*, *Mdh1* and *Eno1* may contribute, in part, to the development of alcohol tolerance. A subsequent study by Eanes et al (2009) confirmed

these findings, where they observed that significant increases in alcohol tolerance were correlated with partial reductions in cytosolic MDH activity in a Drosophila model. Together, these findings suggest that the development of alcohol tolerance is associated with decreased levels of *Mdh1* and *Eno1*. In our current study, we utilised the alcohol-preferring C57BL/6 mouse strain for both exposure groups (ethanol and sucrose control). However, it has been shown that alcohol tolerance pathways are activated upon alcohol exposure (Gordon and Doyle, 1986). Thus, although both exposure groups utilised the alcohol-preferring C57BL/6 mouse strain, alcohol tolerance pathways would likely have only been activated in the ethanol exposed group. This in turn may have decreased the expression of *Mdh1* and *Eno1* in ethanol exposed males (possibly via an epigenetic/DNA methylation mechanism). It is therefore interesting to note that we observed decreases in *Mdh1* and *Eno1* expression in the brains of male embryos sired by ethanol exposed fathers (although we did observe a decrease in *Pgk1* expression, which is contradictory to associations with alcohol tolerance). These changes in gene expression may be evident of an enhanced alcohol tolerance phenotype, passed on by their sires. It is therefore possible that this is further evidence of paternally transmitted foetal programming (that is, programming of the paternal germline in response to an environmental exposure that transmits an adaptive phenotype to their offspring). This has, in part, been supported by findings that sons and daughters of alcoholics demonstrate a low alcohol response, which predisposes them to alcoholism (abuse or dependence) later in life (Carpenter et al., 1991; Schuckit, 1996).

3.4.3.3. Homeostasis is broadly affected by alcohol exposure and may be responsible for growth restricted phenotype in embryo brains

Our GO analysis of expression data from offspring brains revealed enrichment for various biological processes related to homeostasis (*homeostasis*, *cellular homeostasis*, and *cellular redox homeostasis*). The genes associated with biological processes related to homeostasis (*Apoa2*, *Gjd2*, *Id2*, *Prdx5*, *Glrx3*, *Prkcb*, *Txndc12*, *Sp1*, *Trf*) were all downregulated in the brain. The apparent alteration of energy metabolism due to alcohol exposure described above provides a quintessential example of how alcohol can elicit an effect on a homeostasis system. Further to this, alcohol exposure has been associated with various other forms of homeostasis dysregulation. Alcohol exposure has been associated with broadly altered transcriptional activity and homeostasis in the brain (Covarrubias et al., 2005), where 341 genes were upregulated, and 234 downregulated. Alcohol exposure has also been associated with altered homeostasis of N-methyl-d-aspartate (NMDA) receptor regulation which leads to enlargement of dendritic spines of the mouse hippocampus (Carpenter-Hyland and Chandler, 2006). Prenatal exposure to alcohol has been associated with disrupted glutathione homeostasis in the human lung, which is likely to increase susceptibility to acute respiratory distress syndrome (Moss and Burnham, 2003); insulin and IGF resistance and impairs acetylcholine homeostasis in the brain (Soscia et al., 2006); dysregulation of cholesterol homeostasis in the developing brain (Guizzetti and Costa, 2007); and upregulation of foetal cortical neuron glutathione homeostasis which regulates astrocyte development (Rathinam et al., 2006).

Prenatal neural development during the second trimester of gestation is accompanied by formation of the much of the ultimate ectoderm. During this period the neural tube

develops from the elementary ectoderm, and the lumen of the neural tube goes on to develop into the ventricular system of the brain. Prenatal neural dysregulation can be caused by genetic factors and environmental exposures that include influenza virus infection, increased psychological distress, and famine (Chotpitayasunondh et al., 2005). Disruptions of ectodermal development are referred to as dermatoglyphic anomalies, and are therefore indicative of disruptions of prenatal development during the third trimester. Dermatoglyphic anomalies are characterised into two categories: (1) qualitative and/or quantitative differences in finger and palmar ridge formation characteristics and (2) fluctuating dermatoglyphic asymmetries (Chok et al., 2005). A study by Wilber et al (1993) on 22 FAS and nine FAE (foetal alcohol effects – a milder form of FAS) patients revealed that dermatoglyphic asymmetry increased from controls through FAE patients to FAS patients, who demonstrated the greatest dermatoglyphic asymmetry (p=0.0066). From this, these authors concluded that alcohol exposure increased decanalisation of prenatal development.

Chronic alcoholism has been known to cause adaptive changes in neuronal function which can manifest as tolerance, physical dependence and addiction. Messing et al (1986) observed that exposure of cultured neural crest cells to ethanol induced calcium (Ca^{++}) intake, and concluded that cellular adaption to ethanol may involve altered calcium channel regulation. Subsequent studies have provided support for this association. Reynolds et al (1990) found that rats acutely exposed to ethanol demonstrated behavioural tolerance (tolerance to ethanol exposure as measured by performance on rotating rod apparatus) to ethanol which was associated with adaptive changes in the electrophysiological responses of hippocampal CA1 neurons. Moreover, these affects were suggested to have been caused by an inhibitory effect of alcohol on one or more of the mechanisms involved in cytosolic buffering of intracellular calcium Ca^{++}. Gruol and Parsons (1994) demonstrated that chronic exposure to alcohol during development alters the calcium currents of cultured cerebellar

Purkinje neurons. They observed that alcohol-treated neurons were 25% smaller than control neurons, and exhibited lower calcium currents. These authors predicted that alterations of calcium currents by alcohol could have multiple effects on the developing Purkinje neurons and may contribute to the structural and functional changes observed in animal models of foetal alcohol syndrome. This group later observed similar effects of alcohol exposure in developing cerebellar neurons (Gruol and Parsons, 1996). Subsequently, Nagy et al (2000) investigated the effect of alcohol on calcium homeostasis in human lymphocytes. They observed that acute ethanol treatment induced a rapid, transient increase in intracellular calcium concentration in a dose-dependent manner, and concluded that alcohol dependence can develop at the cellular level, and that changes in calcium homeostasis, likely due to direct effects of ethanol on ion channels, may play a central role in processes leading to adaptation of cells to alcohol. More recently, Ren et al (2002) demonstrated that prenatal ethanol exposure alters myocardial contractile function which may contribute to the development of postnatal cardiac dysfunction through increased intracellular Ca^{++} loading and apoptosis.

Overall, our current study observed enrichment of BPs related to homeostasis. That is, genes which demonstrated significantly differential expression in the brains of embryos sired by ethanol exposed males when compared to controls are partly involved with homeostasis during brain development. There is extensive support for the role of homeostasis in the brain during foetal development, where alcohol prenatal exposure has been shown to dysregulate neural homeostasis, which can lead to apoptosis. As apoptosis is a hallmark feature of foetal alcohol syndrome, the aetiology of the programmed cell death observed in FAS, may in part be explained by homeostasis dysregulation (possibly via altered calcium channel activity).

Sp1 is a zinc finger transcription factor protein that binds to GC rich sequence motifs, including promoter regions. As a transcription factor, Sp1 regulates a variety of genes involved in cellular differentiation and apoptosis, and has also been associated with the regulation of copper homeostasis (Liang et al., 2012; Song et al., 2008) and thymic homeostasis (List et al., 2002). Moreover, Sp1 has been shown to play a role in calcium-regulated myocardial gene expression (McDonough et al., 1997). Subsequent to this finding, Zawia et al (1998) noted that PC12 cells cultured in the presence of lead, zinc or cadmium prematurely enhanced the binding of Sp1, which dysregulated expression of genes encoding myelin basic protein and proteolipid protein. They further demonstrated this effect in the cerebellum of rats exposed to lead during the neonatal period. These authors therefore demonstrated metal-induced perturbations of transcriptional regulation of genes that play a role in brain development, as a consequence of aberrantly enhanced Sp1 binding activity.

In the current study, it was observed that the whole brain weight of male embryos sired by ethanol exposed males was significantly decreased when compared to controls. Furthermore, *Sp1* was shown to be significantly differentially expressed in the brains of these embryos, and clustered within the *homeostasis* biological process in our gene ontology analysis. These findings may allude to a mechanism of the observed brain growth retardation. It may be plausible that this growth restriction phenotype was a result of initial *Sp1* dysregulation in the embryonic brain, which consequently lead to altered calcium channel activity, and apoptosis (which lead to decreased brain weight). This proposed mechanism that causes a reduced brain weight phenotype, is synonymous with that of FAS.

From our gene ontology analysis, it was further observed that *Sp1* also clustered in the *homeostatic process* biological process, along with *inhibitor of DNA binding 2* (*Id2*). Both of these genes were shown to be significantly downregulated in the brain compared to

expression in control brains. The Id family of proteins act antagonistically to basic helix-loop-helix (bHLH) proteins. They are able to bind the ubiquitously expressed bHLH E-proteins or cell lineage-restricted bHLH transcription factors, leading to inhibition of lineage-specific gene expression and differentiation (Amat et al., 1998). These proteins mediate mitogenic signals, inhibit differentiation, and play a role in animal development (Rusinova, 2003). In particular, *Id2* plays a role in neural development, where it acts as an antagonist of neuronal differentiation (Gleichmann et al., 2002) and functions in dendritic development (Hacker et al., 2003). Park et al (2013) observed that mice with elevated *Id2* expression during embryonic stages develop precocious neural stem cell depletion and abnormal brain development (microcephaly). *Id2* overexpression has also been shown to arrest differentiation in a variety of in vitro cell line models and enhance apoptosis (Florio et al., 1998). Further to this, Id2 and Id3 have been shown to be important regulators of cellular proliferation and transdifferentiation downstream of TGF-B pathways, including BMP-7 (Kowanetz et al., 2004). More recently, Mathew et al (2013) demonstrated that ablation of *Id2* altered circadian feeding behaviour (and associated locomotor activity), sex-specific enhancement of insulin activity (increased glucose tolerance and insulin insensitivity in males), and elevated glucose uptake in skeletal muscle and brown adipose tissue. These studies provide further support for the findings in our study, which observed significant decreases in *Id2* expression in the brains of embryos of alcohol exposed males, which was associated with decreased brain weights in male embryos, where enrichment of energy (glucose) metabolism biological processes were evident.

Preconception paternal alcohol exposure may be associated with postnatal neurobehavioural abnormalities

The GO analysis conducted in the current study revealed the possibility that preconception paternal alcohol exposure may lead to an increased susceptibility of dementia in sired offspring. This is evident from the functional annotation clustering analysis (using DAVID) of differentially expressed genes in the brain, which were suggestively enriched for dementia diseases (Figure 46).

Functional annotation clustering

Annotation Cluster 4	Enrichment Score: 0.87			Count	P_Value	Benjamini
GOTERM_CC_FAT	respiratory chain	RT		3	4.1E-2	9.4E-1
KEGG_PATHWAY	Huntington's disease	RT		4	9.0E-2	8.8E-1
KEGG_PATHWAY	Parkinson's disease	RT		3	1.7E-1	9.1E-1
SP_PIR_KEYWORDS	mitochondrion inner membrane	RT		3	2.6E-1	9.5E-1
KEGG_PATHWAY	Alzheimer's disease	RT		3	2.7E-1	9.7E-1

Figure 46: Functional annotation clustering analysis using DAVID of differentially expressed genes in the E16.5 brain.

Furthermore, *Apoa2*, which was found to be one of the top differentially expressed genes in the brain, with the highest fold change (FC = 2.55, p=6.27E-05), has been previously reported to be associated with dementia related diseases. ApoA2 is the second most abundant protein of serum high-density lipoprotein (HDL). The level of ApoA2 in plasma was found to be significantly lower (p=0.003) in MCI (mild cognitive decline) patients (n=257) than in age-matched non-demented individuals (n=407) (Song et al., 2012). Consistently, it has been found that Japanese patients with late-onset non-familial Alzheimer's disease have significantly lowered plasma levels of ApoA1 and ApoA2 (Kawano et al., 1995). The gene was found to be downregulated in brain samples of E16.5 embryos when comparing the ethanol

treated sire group with controls (FC = 2.55, *p*=0.00006). Lastly, the differential expression of *Pon1* in the liver may also be suggestive of a contributor to dementia. It has previously been shown that Pon1 downregulation in the mouse liver is associated with hyperhomocysteinemia (Janel et al., 2004), and elevated plasma homocysteine is a known risk factor for cardiovascular disease and thrombosis (Cattaneo et al., 1998). Furthermore, it has been shown that elevated homocysteine levels have been found to be associated with Alzheimer's disease (Morris, 2003; Smith et al., 2010).

Further to this dermatoglyphic anomalies have been identified in FAS patients, as discussed previously. Dermatoglyphic asymmetry has also been associated with mental retardation, borderline personality disorder (Jelovac et al., 1998), autism (Walker, 1977) and schizophrenia (Chok et al., 2005). Paternal contributions to schizophrenia have also been reported, where impairments in imprinting have been highlighted as possible contributing factors (Malaspina, 2001). Together, these findings suggest that preconception paternal alcohol exposure may be associated with mental deficits in their offspring.

3.4.3.4. Preconception paternal alcohol exposure may impact on embryonic development

Arguably the most relevant biological process enriched in the gene ontology analysis of the current study, which supports the hypothesis that preconception paternal alcohol exposure is able to elicit a phenotypic effect in sired offspring, is *embryonic development ending in birth or egg hatching*. The genes that clustered within this biological process were *Distal-Less Homeo Box 2* (*Dlx2*); *LOC100044170* (a hypothetical protein-coding gene); *Eno1*; *serpin peptidase inhibitor clade A (alpha-1 antiproteinase, antitrypsin) member 1b* (*Serpina1b*); *Sp1;* and *transcription factor 7-like 2* (*T-cell specific, HMG-box*) (*Tcf7l2*). In addition to the

contributions of *Eno1* and *Sp1* discussed above *Dlx2* is also known to contribute to foetal development.

Members of the *Dlx* gene family contain a homeobox that is related to that of *Distal-less* (*Dll*), a gene expressed in the head and limbs of the developing fruit fly (Cohen et al., 1989). The DLX proteins are thought to play a role in forebrain and craniofacial development, where *Dlx1* and *Dlx2* are co-expressed in the E12.6 mouse forebrain (Bulfone et al., 1993). Loss of expression of *Dlx1* and *Dlx2* (encoding distal-less homeobox 1 and 2) in the ganglionic eminence results in loss of all detectable migration from the ganglionic eminence to the neocortex (Anderson et al., 1997). Further to this, Qui et al (1995) observed that mutations of *Dlx1* and *Dlx2* produced malformations of the skull in E16.5 mouse embryos, which included that of the craniofacial bones and cartilages. Furthermore, these skeletal abnormalities were associated with abnormalities of the vasculature, peripheral nervous system, and muscles adjacent to dysmorphic bones.

Retinoic acid (RA) has been shown to be indispensable for normal morphogenesis and organogenesis of most vertebrate species. However, RA administration during foetal development has been shown to act as a potent teratogen (Escriva et al., 2002). In a study by Duester et al (1991), foetal alcohol syndrome appeared to be mediated through retinoic acid (RA) signalling. These authors concluded this as they observed that ethanol competitively inhibits retinol and retinaldehyde dehydrogenases in some tissues, while causing elevated RA levels in other tissues. In the periocular mesenchyme, as well as in the pharyngeal arches, the effects of the paracrine RA signal gradients are known to regulate important gene expression and signalling pathways, including *Dlx* genes (Ellies et al., 1997). Ellies et al observed that craniofacial cartilage dysmorphogenesis coincided with a loss of *Dlx* gene expression in retinoic acid-treated zebrafish embryos. Further to this, Vieux-Rochas et al

(2007) demonstrated that mouse embryos exposed to RA during prenatal development induced craniofacial malformations, particularly that of the jaw and cartilage, as a consequence of *Dlx2* repression. More recently, Kleiber et al (2012) exposed mice to 5g/kg of ethanol in utero, and examined long-term changes in gene expression of the offspring brain transcriptome. These authors observed an enrichment for GO functional annotations that were related to neuronal migration and differentiation, which were significantly associated with altered expression of *Dlx1* and *Dlx2*. Together, these studies provide evidence that *Dlx2* may be involved in the aetiology of FAS, which may be synonymous with the paternally mediated effect observed in this study.

3.4.4. Biological processes identified by Gene Ontology analysis of differential gene expression in embryo liver

Gene ontology analysis revealed a number of biological processes that were enriched for in genes which showed a significant change in expression in the livers of embryos sired by ethanol treated males when compared to control embryos. These biological processes included: *response to wound healing*; *chitin catabolic process*; *chitin metabolic process*; *aminoglycan catabolic process*; *inflammatory response*; *polysaccharide catabolic process*; *nitrogen compound biosynthetic process*; *glutamine family amino acid metabolic process*; and *aminoglycan metabolic process*. These biological processes were broadly categorised into three categories: response to wound healing; aminoglycan metabolism (encapsulating *aminogylcan metabolic process*; *aminoglycan catabolic process*; *chitin metabolic process*; *chitin catabolic process*; *polysaccharide catabolism; nitrogen compound biosynthetic process*); and inflammatory response.

3.4.4.1. Response to wound healing may be related to diverted arginine metabolism

The biological process *wound healing* was enriched in our gene ontology analysis of the genes shown to be downregulated in embryonic livers of mice sired by ethanol treated males (Figure 44). Gene ontology term was comprised of the genes *Arg1*, *Chi3l4*, *C8g*, and *Chi3l3* (Table 16).

Wound healing is a complex process that is initiated in order to restore tissue damage. Arginine has been shown to be an important factor in wound healing (Barbul, 1990; Curran et al., 2006; Sax, 1994; Witte et al., 2003) via its catabolism to nitric oxide via NOS. This was initially identified in neurons and endothelial cells, where these cells were stimulated to synthesise nitric oxide (NO) through calcium signalling (Klatt et al., 1992). Arginine obtained from the diet is metabolised by two means. Firstly, arginine can be catabolised by nitrogen oxide synthase (NOS) to form citrulline and NO, where NO is involved in aspects that accompany wound healing, including, immune responses, angiogenesis, and establishment of new epithelium (Debats et al., 2009). Alternatively, arginine can be metabolised by arginase (Figure 47). Arginase metabolism is mediated by two isoforms: namely, arginase-1 (Arg1) and arginase-2 (Arg2). Arg1 is the liver, cytosolic form, and Arg2 the mitochondrial isoform located in the kidney, prostate, small intestine and breast. Arginase catalyses the conversion of arginine to ornithine and urea, where ornithine is used as an essential precursor to collagen and polyamine synthesis, which are required for wound healing (Morris, 1992).

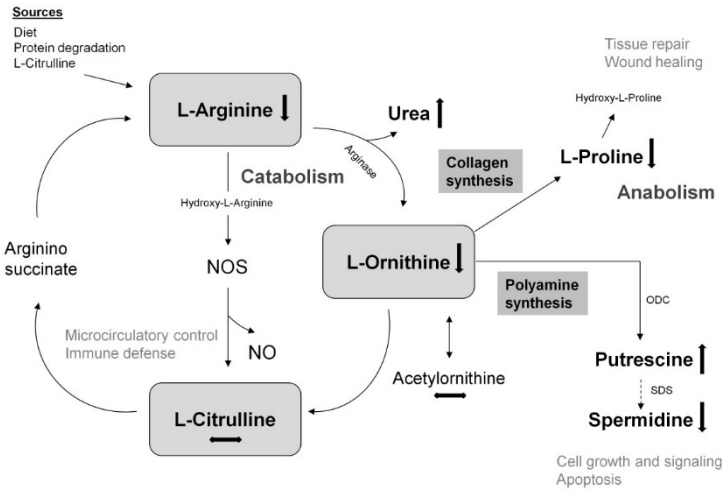

Figure 47: Arginine metabolism. http://ccforum.com/content/figures/cc11205-1-l.jpg

Previous animal and in vitro studies have demonstrated that *Arginase 1* (*Arg1*) is upregulated during normal wound healing, which is needed for collagen formation (Thornton et al., 1998). Debats et al (2009) further observed in a human study, that ARG1 levels were significantly higher within wound fluid than in plasma, supporting previous animal findings that arginine plays a role in wound healing responses of the skin.

Overexpression of NOS has been associated with the inhibition of post-injury neural regeneration (Mesenge et al., 1996). De la Monte et al (2003) further demonstrated that the overexpression of NOS3 causes neuronal death (as a consequence of apoptosis) and impairs neuronal mitochondrial function, which may be relevant to neuro-degeneration in Alzheimer's disease. While it has been shown that NO has the ability to protect cells against apoptosis (Kim et al., 1999; Liaudet et al., 2000), NO-mediated apoptosis has also been observed (Liaudet et al., 2000; Shen et al., 1998). This effect has been noted in macrophages (Albina et al., 1993), vascular epithelial cells (Lopez-Collazo et al., 1997) and ventricular myocytes (Pinsky et al., 1999). The latter observation may account for the fatal

cardiovascular depression induced by overproduction of nitric oxide in rostral ventrolateral medulla of the rat (Chan et al., 2002). NO-induced apoptosis can progress to necrosis if it persists to such an extent that sufficient cellular stores of high-energy phosphates are depleted (which is required for apoptosis) (Bonfoco et al., 1995; Leist et al., 1999).

Our study observed that *Arg1* was downregulated in the liver of embryos sired by ethanol treated males (FC=1.61, *p*=3.87E-05). Considering that arginine can be metabolised by either Arg1 to ornithine, or NOS to NO, it may be plausible to predict that if Arg1 activity was reduced, the majority of arginine would be diverted down the NOS catabolic pathway and significantly increase the presence of NO. Studies of arginine metabolism by activated macrophages noted that the majority of arginine is consumed for the production of urea rather than NO. Furthermore, it was found that inhibition of arginase results in increased NO production (Chang et al., 1998; Hey et al., 1997; Tenu et al., 1999), particularly in endothelial cells (Chicoine et al., 2004). These findings therefore suggest that reduced *Arg1* expression may divert arginine metabolism, from ornithine production to citrulline production, and increase the production of NO (Figure 47).

Together, these results demonstrate that decreased Arg1 levels (decreased *Arg1* expression), may consequently increase production of NO, which has been associated with apoptosis. This observation may account for the reduced liver weight observed in this study. Reduced liver weight has been observed in a rat model of prenatal alcohol exposure (Henderson et al., 1979). Thus, the observed reduction in liver weight in our current study, may suggest a paternally transmitted foetal alcohol syndrome-like phenotype.

3.4.4.2. Preconception paternal alcohol exposure has minimal effect on embryonic placental gene expression

The small number of significantly differentially expressed genes in the placenta was much lower than that observed in the brain and liver, and might suggest that the placenta as a tissue is more resilient to the effects of alcohol exposure. Furthermore, we observed not significant differences in placental weight of embryos sired by ethanol treated males when compared to those of controls. The effect of prenatal exposure on placental weight is conflicted. Haycock and Ramsay (2009) observed a significant decrease in placental weight of mice prenatally exposed to ethanol, while other studies have noted increases in placental weight associated with in utero alcohol exposure (Aufrere and Le Bourhis, 1987).

Abel (1995) examined the effects of ethanol exposure of male rats and the outcome of their progeny. This study used two treatment groups: one group exposed to 5g/kg ethanol for 3 weeks (exposing sperm during their latter, postmeiotic period of maturation from early spermatid to mature spermatozoa); and a second group exposed for 9 weeks (exposing sperm to alcohol throughout their maturation, prior to and after maturation). They noted that offspring placental weight remained unchanged in the first exposure group, while the second group showed an increase in placental weight. These findings were not observed in our study. However, the dosage used in the present study was 3g/kg, while that of Abel was 5g/kg.

Despite the lack of enrichment for biological processes in the placental gene ontology analysis, there were four genes which showed significantly reduced expression levels (*Rapgef3*, *Unc45a*, *Unc5b*, and *Wdfy1*), and one gene showed downregulation (*Gzmd*) in the placentae of embryos of ethanol treated sires. A study by Rosenberg et al (2010)

investigated the effects of moderate drinking (2.82g/kg ethanol per day) during pregnancy on placental gene expression in rats. Using a microarray analysis of 28,000 genes, the observed 304 genes that showed a minimum of twofold change in expression at a significance cut-off at p=0.05. Of these genes, 76% were downregulated and 24% upregulated. Although based on a limited number of genes, our data also demonstrates ~80% (4/5) of genes significantly differentially expressed in the placenta are downregulated, and ~20% upregulated (1/5). However, of these genes significantly down- and upregulated in, placentae none of the genes observed in our study overlapped with those of Rosenberg et al. From this, we conclude that preconception paternal alcohol exposure has a minimal effect on placental gene expression of sired embryos.

In summary, our gene ontology analysis of gene expression profiles observed in the brain and liver of embryos sired by ethanol treated males, reveal that the majority of the biological processes enriched in these expression profiles, together with the biological functions of the genes that cluster within them, are related to foetal alcohol syndrome-like phenotypes. These include skeletal malformations, immune dysfunction and apoptosis in the brain and liver. Enrichment of apoptosis in the brain GO analysis was also associated with reduced brain weight in male offspring. Due to the fact that offspring (embryos) were not directly exposed to alcohol, we conclude that paternal chronic alcohol exposure prior to conception can alter gene expression in sired offspring, and elicit a growth restriction phenotype (as evidenced by the reduced foetal brain and liver weight), that is synonymous with FAS.

Chapter 4

Alcohol-induced sperm DNA methylation changes are associated with altered gene expression and phenotype in offspring

The current study aimed to investigate the effects of chronic preconception paternal alcohol exposure on the sperm methylome. It further aimed to determine whether alterations in DNA methylation levels at a given gene in the sperm, was associated with a change in gene expression in sired offspring. This logic is based on the assumption that aberrant DNA methylation profiles are transmissible through the paternal germline via the mature sperm, and may alter epigenetic control of gene expression (Figure 48). This in turn, may result in a dysregulated gene state in offspring, and contribute to the manifestation of a phenotype associated with FAS.

In our current study, we assessed DNA methylation levels within the sperm of alcohol exposed sires (but not in the tissues of sired offspring), and determined whether this was ultimately associated with a change in embryonic gene expression. Thus, while we did not attain embryonic DNA methylation data to investigate direct evidence for epigenetic inheritance of aberrant DNA methylation states from the sperm, we inferred that gene expression alterations are as a consequence of changes in underlying epigenetic regulatory mechanisms. Therefore, alterations in gene expression may be related to inherited changes in DNA methylation which influenced regulation of gene expression in embryonic tissues.

The present study hypothesised that significant alterations in sperm DNA methylation at a given gene in parallel with observed changes in gene expression observed in embryonic tissues, were as a result of the inheritance of abnormal DNA methylation levels by offspring, which lead to an altered expression of that given gene (Figure 48).

As the current study only produced offspring (embryos) from the CAM model, investigations into whether genes demonstrating significant changes in sperm DNA methylation of treated sires were associated with concomitant altered expression patterns in offspring, only the CAM sperm DNA methylation data was used to compare against gene expression in their sired offspring.

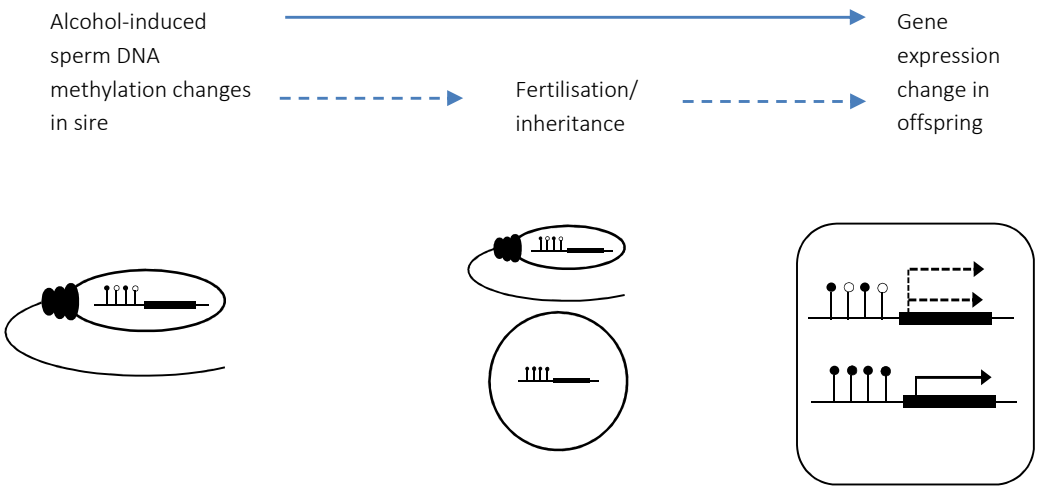

Figure 48: Study model to explore the hypothesis that changes in gene expression in embryonic tissue are consequential to inherited alterations in DNA methylation. This model illustrates the manner by which the present study investigated the effect of preconception paternal alcohol exposure on sperm DNA methylation, and alterations in gene expression of sired offspring (square box). The solid blue arrow shows that only sperm DNA methylation and gene expression in offspring was tested in the present study. The dashed blue arrows show that the mouse model employed in the current study inferred that if changes in offspring (embryo) gene expression were observed, it was likely due to alcohol-induced DNA methylation aberrations that were inherited from treated sires. Dashed black arrows depict a deviation in normal (wild-type) gene expression (overexpressed or underexpressed) from the paternal allele; the solid black arrow depicts normal gene expression from the maternal allele (which was inherited from the unexposed oocyte (circle).

4.1. Evidence for DNA methylation changes in the sperm of ethanol exposed mice with associated dysregulated expression in offspring tissues

In order to determine whether there was evidence of altered DNA methylation from the sperm RRBS data that was associated with a concomitant change in gene expression in the tissues of sired offspring, an overlap analysis was performed. That is, we investigated whether the 266 common significant DMRs identified in the CAM model (ETOH1/ETOH2 common DMRs) RRBS sperm DNA methylation data were associated with changes in embryonic expression of genes that were in close proximity to a DMR. Of the 266 DMRs, 224 were within 100kb of a unique known/annotated gene. Of these, 99 were observed to be expressed in the brain, 74 in the liver and 84 in the foetal placenta (Figure 49). Of these, a total of 38 genes showed significant differential gene expression (p<0.05) (Table 17), whose position was located adjacent to a common significant DMR observed in the RRBS sperm DNA methylation data (p-value calculated using LIMMA (Smyth et al., 2002)). This evidence suggests that alcohol-induced epimutations in the sperm appear to have been transmitted to sired offspring, as reflected in the dysregulated gene expression in embryonic tissues.

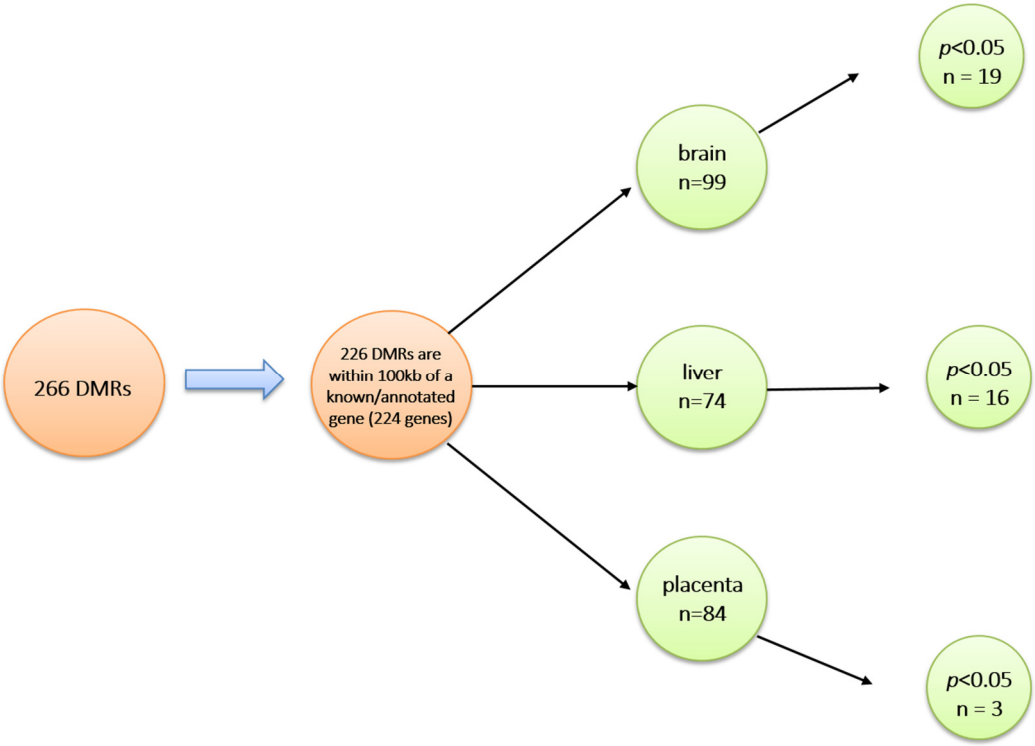

Figure 49: Overlap analysis of DMRs with genes demonstrating significant changes in expression. Of the 266 common significant DMRs observed in the CAM model RRBS sperm DNA methylation data, 226 DMRs were located within 100kb of a known/annotated gene, and 224 were uniquely located within 100kb of a known/annotated gene. Of these 224 genes, the expression of 99, 74 and 84 were observed in the foetal brain, liver and placenta, respectively. Of these, 19, 16 and 3 genes demonstrated a significant change in gene expression ($p<0.05$, total=38).

Table 17: Genes demonstrating an overlap in altered DNA methylation in sperm and expression in embryonic tissues

Gene	Direction of gene expression change	*p*-value	Brain FC	Direction of DNA methylation change	% DNA methylation difference
Id4	Down	0.0013076	1.8825323	HYPO	11.40
C530008M17Rik	Down	0.0047676	1.4538612	HYPO	12.20
Tox3	Down	0.0053795	1.6433825	HYPO	10.70
C80913	Down	0.0078071	1.2436135	HYPER	14.50
Grm7	Down	0.0088587	1.246976	HYPER	19.40
Tgfb2	Down	0.0094814	1.2204749	HYPO	29.45
Zfp317	Down	0.0183215	1.3327076	HYPO	12.10
Depdc1b	Up	0.0208717	1.176778	HYPER	13.40
Gm1943	Down	0.0211978	1.122127	HYPER	11.10
Psmg2	Up	0.0232167	1.1631146	HYPER	12.35
Vwf	Up	0.0245708	1.1649306	HYPO	18.35
Dcakd	Up	0.0320537	1.1639182	HYPO	13.90
2900026A02Rik	Up	0.0361836	1.1659031	HYPO	13.85
Itgb5	Down	0.0369522	1.2050741	HYPO	11.05
Sez6	Up	0.0388832	1.1438989	HYPO	14.00
1700020O03Rik	Down	0.0405525	1.2265146	HYPO	11.10
Odc1	Down	0.0439164	1.1414818	HYPO	14.30
Ccnd3	Up	0.0464967	1.1220971	HYPO	22.75
Igf1r	Up	0.0496649	1.1780038	HYPO	12.35

Gene	Direction of gene expression change	*p*-value	Liver FC	Direction of DNA methylation change	% DNA methylation difference
Bcl2l1	Up	0.0023758	1.1812949	HYPO	10.90
Rnf123	Up	0.0043662	1.2052569	HYPO	17.10
Tgfbr3	Up	0.0043881	1.2682375	HYPO	12.00
Xpr1	Up	0.0066132	1.2143603	HYPER	10.45
Igf1r	Up	0.0084032	1.2303873	HYPO	12.35
Lmna	Up	0.0122368	1.2174683	HYPO	17.65
BC030476	Down	0.0151554	1.5070822	HYPO	12.35

Depdc1b	Up	0.0170091	1.1578375	HYPER	13.40
Ggnbp1	Down	0.0240903	1.143491	HYPO	12.05
Vwf	Up	0.0290631	1.2459334	HYPO	18.35
Cdc5l	Up	0.0326117	1.1593753	HYPER	16.25
Slc2a2	Down	0.0396244	1.271498	HYPER	16.75
Pcbp3	Up	0.0396632	1.1032306	HYPO	15.85
Brd7	Up	0.0428862	1.1155279	HYPO	14.70
Bcor	Up	0.0468783	1.148939	HYPO	11.40
Ccnd3	Up	0.0497502	1.1386689	HYPO	22.75

		Placenta			
Gene	**Direction of gene expression change**	***p*-value**	**FC**	**Direction of DNA methylation change**	**% DNA methylation difference**
Ctcfl	Up	0.0142317	1.3259266	HYPER	10.10
Odc1	Up	0.0198184	1.1618588	HYPO	14.30
Akap1	Up	0.0498653	1.1861802	HYPO	16.75

Genes shown in red are common to the brain and liver, and the gene shown in blue is common to the brain and placenta. Up = upregulation of gene expression, down = downregulation of gene expression, FC = fold change in gene expression relative to control, HYPO = hypomethylated (decreased DNA methylation when compared to control), HYPER = hypermethylated (increased DNA methylation when compared to control). Significance levels for the change in DNA methylation are all $p<0.05$.

4.2. Epigenetic alterations in disparate lineages may provide evidence of an inherited epigenetic insult from the male gamete

The overlap analysis conducted in our study further revealed that four genes (*Depdc1b*, *Vwf*, *Ccnd3*, and *Igf1r*) were common to the brain and liver, while only one gene (*Odc1*) was common to the liver and the placenta, where all of these genes were significantly upregulated in embryonic tissues, except for *Ocd1* which was significantly downregulated in the brain (Table 16). This evidence may suggest that epigenetic modifications/regulation of these genes is likely to have been altered prior to differentiation of these lineages, which in light of our hypothesis, would have been expected.

Embryonic stem cells give rise to four cell lineages: the ectoderm (brain, skin), mesoderm (muscle, blood, bone cartilage), endoderm (lung, gut and liver) and the germline (Yabut and Bernstein, 2011). Following fertilisation, the E2.5 mouse embryo contains eight identical cells. At E3.0, the blastomeres which express the transcription factor *OCT3/4* give rise to the inner cell mass (ICM), while those that express *CDX2* give rise to the trophectoderm (TE) of the E3.5 blastocyst (Arnold and Robertson, 2009). The TE ultimately gives rise to the placenta, while the inner cell mass (ICM) gives rise to the embryo proper. Cells of the ICM of the blastocyst that express the transcription factor *GATA6*, give rise to the primitive endoderm, while those that express *Nanog* give rise to the epiblast of the late blastocyst at E4.5 (Arnold and Robertson, 2009). At E6.5-7.5, gastrulation of the embryo occurs. Cells of the epiblast converge towards the posterior of the embryo as the primitive streak, and give rise to the embryonic and extraembryonic mesoderm. As the primitive streak expands to the distal tip of the embryo, cells that are present in the anterior primitive streak give rise to the definitive endoderm. Cells that

remain in the epiblast cell layer by the end of gastrulation constitute the neuroectoderm (Arnold and Robertson, 2009).

Thus, the placental tissue cell lineage and those of the embryonic organs (brain and liver) are defined at E3.0 in the mouse, and diverge by E3.5. Furthermore, the endoderm (liver lineage) and ectoderm (brain lineage) are both derived from the epiblast during gastrulation (E6.5-7.5). In our current study, we observed significant changes in gene expression of *Depdc1b*, *Vwf*, *Ccnd3*, and *Igf1r* in both the embryonic brain and liver of embryos sired by ethanol exposed males. This may suggest that the epigenetic mechanisms that govern the expression of these genes were altered in the epiblast, which was subsequently propagated during differentiation into the ectoderm (brain) and endoderm (liver). Moreover, we observed a significant change in *Odc1* expression in both the liver and the placenta of E16.5 embryos. This finding may suggest that epigenetic mechanisms that regulate *Odc1* expression were altered before the differentiation of the ICM and TE (i.e. at conception), and therefore present in the E2.5 embryo. It may therefore be plausible that the epigenetic aberration of *Odc1* was inherited from the gametes that formed the early embryo. As only male mice were exposed to alcohol in the CAM model (females were untreated and thus wild-type), it may be reasonable to conclude that the abnormal epigenetic (DNA methylation) state at *Odc1* which was present in the sperm of chronically exposed males, was inherited by the embryo during fertilisation, consequently propagated through both the placental (TE) and liver (ICM) cell lineages, and dysregulated *Odc1* expression in these tissues of the E16.5 embryo. This finding supports our hypothesis whereby an alcohol-induced epigenetic insult is transmitted via the sperm to sired offspring, and mitotically inherited through cellular division in the developing embryo, which is associated with a change in gene expression.

4.3. Epimutations may be recovered in a tissue-specific manner

The overlap analysis presented in this chapter reveals that some genes that show both a change in sperm DNA methylation and embryonic expression tissues are common to two tissues. For example, *Igf1r* is common to the brain and liver. The explanation provided in Section 4.1.2. above argues that this is possibly explained by an early epimutation (inherited from the sperm of alcohol exposed males) that is propagated and stably inherited during mitotic divisions in the developing embryo, and because the epigenetic status of such a gene is altered, leads to a change in gene expression. Based on this reasoning, one might expect that the epimutation is propagated and inherited in all cell lineages, and thus it would be expected to demonstrate altered gene expression in all embryonic tissues (brain, liver and placenta). Thus, the alcohol-induced *Igf1r* epimutation (decreased DNA methylation in the sperm) would have been transmitted to the placental tissue, where we would expect a similar change in gene expression as that observed in the brain and liver. However, *Igf1r* expression did not appear to be changed in the placenta. Moreover, the change in gene expression was not of the same magnitude in the brain and liver (FC=1.18 v. FC=1.30, respectively, albeit that these changes are very similar). This might suggest that the placenta was able to recover from the epimutation, and restore *Igf1r* expression. Thus, *Igf1r* expression may have been dysregulated in all embryonic cell lineages, but following cell lineage differentiation into E16.5 embryonic tissues, *Igf1r* expression was restored due to possible recovery from epigenetic changes (as with *Depdc1b*, *Vwf*, and *Ccnd3*). Similarly, *Odc1* was common to the brain and placenta, but not the liver, suggesting that the embryonic liver tissue was able to recover from the epimutation and *Odc1* gene expression was restored to a wild-type level, and therefore not significantly altered in the liver. Overall, this might suggest some embryonic tissues are able to recover from epigenetic aberrations and restore wild-type tissue specific expression.

4.4. Preconception paternal alcohol exposure is associated with changes in gene expression related to growth and neurodevelopmental phenotypes

The overlap analysis conducted in this study revealed that 38 genes demonstrated a significant change in sperm DNA methylation, and a significant change in gene expression in offspring sired by ethanol exposed males. Of these, five were common to more than one tissue (*Depdc1b*, *Vwf*, *Ccnd3*, *Igf1r*, and *Odc1*). Using the Mouse Genome Informatics' gene expression database (Smith et al., 2014b), we investigated the embryonic regions in which these five genes were expressed, as well as the any abnormal phenotypes associated with the biological systems to which these genes contribute (Table 18). From this, we observed that three of these five genes (*Ccnd3*, *Igf1r*, and *Odc1*) are expressed in several brain regions of the developing embryo, and three appear to be involved in embryonic limb development (*Depdc1b*, *Igf1r*, and *Odc1*).

Growth restriction and mental retardation are hallmarks of FAS, and are two of the diagnostic requirements for the diagnosis of the syndrome (CDC, 2004). Numerous animal and human studies have demonstrated that prenatal exposure to alcohol results in intrauterine growth restriction (IUGR), decreased birth and brain weight, and CNS abnormalities (Carter et al., 2013). Structural abnormalities of the brain include reduction in size, or altered shape of the corpus callosum, cerebellum, or basal ganglia (CDC, 2004), which in turn can be a contributing factor to growth deficiencies, where CNS damage can affect skeletal growth. Skeletal abnormalities associated with FAS include vertebra and rib anomalies, and short metacarpal bones (CDC, 2004). These prenatal growth and CNS deficits persist into adulthood (Carter et al., 2007; Day et al., 1999; Jacobson et al., 1994; Streissguth, 1990). These phenotypes are strongly aligned with the regions of embryonic

expression, and several abnormal phenotypes associated with the five overlap genes common to more than one tissue (Table 18). In addition to the above phenotypes, FASD has been associated with various cancers in children (Burd et al., 2014). These include blastomas (neuro-, hepato-, and nephroblastoma), Hodgkin disease, leukaemia and rhabdomyosarcoma.

Table 18: Genes overlapping in foetal tissues and their association with disease pathology.

	Gene	Function	Regions of expression in embryo*	Abnormal phenotypes reported in these systems	References
Depdc1b	*DEP domain containing protein 1b*	signalling proteins	Limb mesenchyme, genital tubercle	Human: Bladder cancer; breast cancer	(Kanehira et al., 2007) (Katagiri et al., 2009) (Marotta, 2013)
Vwf	*Von Willebrand Factor homolog*	antihaemophilic factor carrier and a platelet-vessel wall mediator in the blood coagulation system. It is crucial to the haemostasis process	Heart, liver, testis	*Mouse: cardiovascular, digestive/alimentary, hematopoietic, homeostasis, immune, nervous system Human: von Willebrand's disease	(Casonato et al., 2007)
Ccnd3	*Cyclin D3*	regulators of cyclin-dependent kinases; functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition; shown to interact with and be involved in the phosphorylation of tumour suppressor protein Rb	Brain (hippocampus, olfactory bulb, hindbrain, midbrain, cerebellum), cardiac muscle tissue, eye, heart, liver	Human: Burkitt Lymphoma, gliomas; ocular adnexal lymphoma; hepatocellular lymphoma; acute myeloid leukaemia; T cell acute lymphoblastic leukaemia	(Schmitz et al., 2012) (Buschges et al., 1999) (Li et al., 2013) (Smith et al., 2005) (Sawai et al., 2012)
Igf1r	*Insulin-Like Growth Factor 1 Receptor*	binds insulin-like growth factor; tyrosine kinase activity; critical role in transformation events; overexpressed in most malignant tissues where it functions as an anti-apoptotic agent by	Inner cell mass, mandible, lung, head, brain (pituitary, cerebral cortex, hippocampus, olfactory bulb, cerebellum,	*Mouse: behaviour, cardiovascular, growth/size, homeostasis, mortality/aging, muscle, respiratory,	(Liu et al., 1993) (Fang et al., 2012) (Abuzzahab et al., 2003) (Kruis et al., 2010) (Peiro et al., 2009)

		enhancing cell survival; mediate pre- and postnatal growth	midbrain), eye, femur, liver, heart, testis, epididymis	nervous system, craniofacial, skeleton Human: Organ hypoplasia; developmental delays in ossification; CNS abnormalities; short stature; intrauterine and postnatal growth retardation; organ malformation; severe mental retardation; type 2 diabetes; breast cancer; multiple myeloma	(Veenma et al., 2010) (Walenkamp et al., 2006) (Choi et al., 2011) (Chng et al., 2006)
Odc1	*Ornithine Decarboxylase 1*	encodes the rate-limiting enzyme of the polyamine biosynthesis pathway which catalyses ornithine to putrescine; essential for growth and development; cell proliferation and differentiation	Inner cell mass, trophectoderm, brain (hindbrain, midbrain, amygdala, cerebral cortex, cerebellum), CNS, cranium, facial bone primordia, hindlimb, forelimb	*Mouse: cellular, embryogenesis, mortality/aging Human: cerebral artery occlusion, colonic adenoma recurrence, colon cancer	(Lee et al., 2011) (Chen et al., 2002)

* Obtained from the Mouse Genome Informatics' gene expression database (Smith et al., 2014a)

4.5. GO analysis of overlapping genes suggests an apoptosis-mediated aetiology for the observed the observed growth-restriction phenotype in embryos sired by alcohol exposed males

In order to assess whether there was functional enrichment in genes which showed both a significant change in sperm DNA methylation and a significant change in embryonic gene expression, a functional enrichment analysis of the genes identified in the overlap analysis (DNA methylation/gene expression) was conducted using DAVID (Table 19 and Table 20). This was only possible for the brain and liver tissues, as there were too few (n=3) placental genes identified in the overlap analysis to perform a functional enrichment analysis. A combined functional enrichment analysis was also performed, which included both brain and liver GO terms (Appendix G). A graphical summary of these findings is shown in Figure 50.

Table 19: GO analysis of brain overlap genes.

GO Term	*p*-value	Genes
GO:0051726~regulation of cell cycle	0.0155	*Psmg2, Ccnd3, Tgfb2*
GO:0006461~protein complex assembly	0.0172	*Vwf, Igf1r, Psmg2*
GO:0070271~protein complex biogenesis	0.0172	*Vwf, Igf1r, Psmg2*
GO:0008283~cell proliferation*	0.0202	*Ccnd3, Id4, Tgfb2*
GO:0042401~biogenic amine biosynthetic process*	0.0257	*Odc1, Tgfb2*
GO:0008284~positive regulation of cell proliferation*	0.0261	*Odc1, Id4, Tgfb2*
GO:0065003~macromolecular complex assembly	0.0351	*Vwf, Igf1r, Psmg2*
GO:0043933~macromolecular complex subunit organization	0.0410	*Vwf, Igf1r, Psmg2*
GO:0042398~cellular amino acid derivative biosynthetic process	0.0419	*Odc1, Tgfb2*
GO:0031589~cell-substrate adhesion	0.0517	*Vwf, Itgb5*
GO:0009309~amine biosynthetic process*	0.0631	*Odc1, Tgfb2*
GO:0051259~protein oligomerization	0.0640	*Vwf, Igf1r*
GO:0043523~regulation of neuron apoptosis*	0.0693	*Grm7, Tgfb2*
GO:0006576~biogenic amine metabolic process*	0.0710	*Odc1, Tgfb2*
GO:0042127~regulation of cell proliferation*	0.0837	*Odc1, Id4, Tgfb2*
GO:0042981~regulation of apoptosis*	0.0873	*Psmg2, Grm7, Tgfb2*
GO:0043067~regulation of programmed cell death*	0.0892	*Psmg2, Grm7, Tgfb2*
GO:0007155~cell adhesion	0.0898	*Vwf, Itgb5, Tgfb2*
GO:0022610~biological adhesion	0.0898	*Vwf, Itgb5, Tgfb2*
GO:0010941~regulation of cell death*	0.0901	*Psmg2, Grm7, Tgfb2*
GO:0042060~wound healing*	0.0976	*Vwf, Tgfb2*

* GO terms related to sperm DNA GO enrichment analysis and/or embryonic gene expression GO enrichment analysis

Table 20: GO analysis of liver overlap genes.

GO Term	*p*-value	Genes
GO:0008283~cell proliferation*	0.0236	*Ccnd3, Tgfbr3, Bcl2l1*
GO:0007283~spermatogenesis*	0.0245	*Lmna, Bcl2l1, Ggnbp1*
GO:0048232~male gamete generation*	0.0245	*Lmna, Bcl2l1, Ggnbp1*
GO:0007276~gamete generation*	0.0398	*Lmna, Bcl2l1, Ggnbp1*
GO:0001889~liver development	0.0415	*Vwf, Tgfbr3*
GO:0019953~sexual reproduction*	0.0528	*Lmna, Bcl2l1, Ggnbp1*
GO:0032504~multicellular organism reproduction	0.0588	*Lmna, Bcl2l1, Ggnbp1*
GO:0048609~reproductive process in a multicellular organism	0.0588	*Lmna, Bcl2l1, Ggnbp1*
GO:0051259~protein oligomerization	0.0692	*Vwf, Igf1r*

* GO terms related to sperm DNA GO enrichment analysis and/or embryonic gene expression GO enrichment analysis

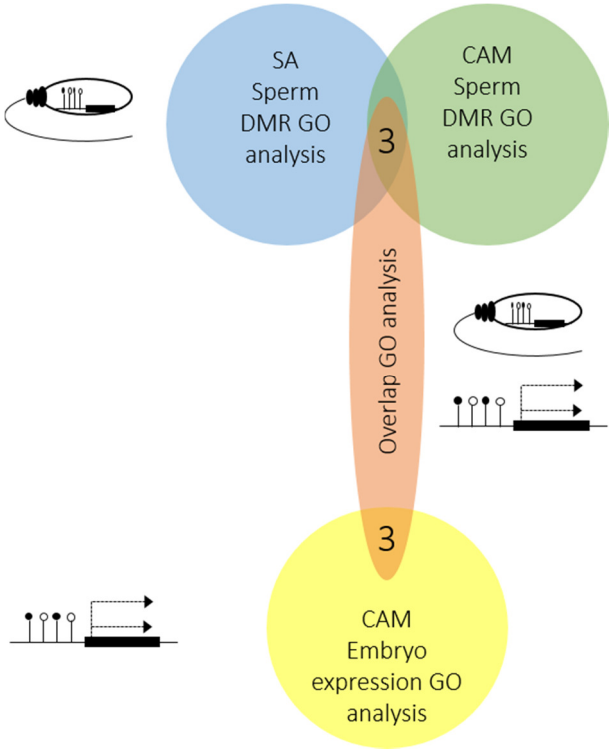

Figure 50: Functional enrichment analysis of genes that showed a significant change in sperm DNA methylation and a significant change in embryonic gene expression and the overlap between the sperm DMR and embryo gene expression GO analyses. A functional enrichment analysis (red ellipse) of genes that demonstrated both a significant change in sperm DNA methylation and embryonic gene expression revealed that a number of biological processes are common to biological processes enriched in the sperm DMR GO analysis of genes common to both biological models (overlap between the SA model in the blue circle and CAM model in the green circle) and embryonic gene expression GO analysis (yellow circle). Three biological processes overlapped with the sperm DMR GO analysis (gamete generation (and spermatogenesis), and cell proliferation and apoptosis); and three biological processes overlapped with the embryonic gene expression GO analysis (growth, membrane component, and wound healing).

Our functional enrichment analysis of the genes identified in the overlap analysis revealed several gene ontology terms/biological processes that were in common with the GO analyses of sperm DNA methylation (DMRs common to both the SA and CAM models) and that performed for embryonic tissue gene expression. Biological processes that overlapped with sperm DMR GO terms included gamete generation (and spermatogenesis), cell proliferation and neural apoptosis (denoted with * in Table 19 and Table 20). Biological processes that overlapped with embryonic tissue gene expression GO terms included embryonic

development (related to growth in Appendix G Annotation Cluster 1 denoted with *), glycan metabolism (related to membrane component in Appendix G Annotation Cluster 5 denoted with *), and wound healing (Table 19).

These findings suggest that biological processes overrepresented in the sperm DMR GO analysis and the embryo gene expression GO analysis are, in part, common to the biological processes enriched in the GO analysis of genes which showed both a significant change in sperm DNA methylation of sires exposed to alcohol prior to conception and which showed a significant change in embryonic gene expression. However, caution must be taken with the GO overlap analysis, as not all biological processes were significant. Thus, these findings may suggest common biological processes that are altered in the sperm DMRs of male mice chronically exposed to alcohol prior to conception, and those altered in sired embryo gene expression. This may also support an apoptosis-mediated/cell proliferation abrogation mechanism for the growth restriction observed in male embryonic brains, which is synonymous with mechanisms related to mental retardation and growth restriction observed in FAS.

4.5.1. *Odc1* downregulation may contribute to the observed reduction in embryonic brain weight

Our overlap analysis revealed that *Odc1* expression was significantly downregulated (p=0.0439) with a concomitant decrease in DNA methylation (14.3%) in the brain of embryos sired by alcohol exposed males (Table 18). This finding is surprising, as reduced DNA is generally associated with increased gene expression. In support of this, Leinonen et al (Leinonen et al., 1987) observed that induced hypomethylation of the human *ODC* gene is

associated with enhanced gene expression. The observed decrease in *Odc1* expression in the current study, may therefore be as a result of an alternative epigenetic mechanism regulating its expression, which was disrupted as a result of paternal alcohol exposure (bearing in mind that neither the DNA methylation nor gene expression data has been validated as yet).

Ethanol has been shown to decrease ornithine decarboxylase (ODC) activity (Thadani et al., 1977). Polyamines, including putrescine, play a fundamental role during embryogenesis by regulating cell growth and proliferation and by interacting with RNA, DNA and protein (Agostinelli et al., 2010). Polyamine biosynthesis is regulated by ODC, where ODC (encoded by *Odc1*) converts ornithine to putrescine (Figure 51). Both acute and chronic ethanol exposure have been found to slow growth and to lower tissue polyamine (putrescine) content (Shibley et al., 1995). Recently, Poodeh et al (2014) demonstrated that mice exposed to 3g/kg ethanol prenatally at E8.75, resulted in a loss of polyamines in the E9.5 head and trunk. This deficiency was associated with slow cell growth, reduction in endothelial cell sprouting, an altered pattern of blood vessel network formation and consequently retarded migration of neural crest cells and growth restriction – a phenotype that is synonymous with that associated with FAS.

Alcohol-induced decrease in ODC activity in embryonic chick embryos results in growth suppression, where both whole embryo and brain weight was reduced (Sandstrom et al., 1993). These authors attributed growth suppression to reduced putrescine content, as exogenous putrescine prevented growth suppression. Prenatal ethanol exposure has also been shown to affect ODC in the brain and heart during foetal development (Thadani et al., 1977), which has been associated with reduced embryo and brain weight (Beeker et al., 1992). In support of these findings, Davidson et al 1998 (1998) observed ethanol-induced inhibition of brain ODC activity in the postnatal rat, and Do et al 2013 (2013) observed

decreased ODC activity in hepatocytes exposed to alcohol in culture, which was associated with decreased cellular proliferation. These authors were also able to demonstrate a reversal of these effects through putrescine supplementation.

In contrast to these findings, Thadani and Schangbergl (1979) observed increased ODC activity in the brain and heart of neonatal rat pups, which were prenatally exposed to alcohol throughout development. This finding is akin to that in our current study, where *Odc1* expression was significantly upregulated (p=0.0198) in the placenta of embryos sired by alcohol exposed males. However, these authors further observed a subsequent decline in ODC activity to normal levels in all parts of the brain, but not the cerebellum, where ODC activity remained elevated. These findings were associated with reduced heart, but not brain weight. From this, it was concluded that prenatal alcohol exposure appears to alter polyamine metabolism and organ weight. Increased ODC activity has been reported during the perinatal and postnatal periods in foetal animals exposed to ethanol during early development. Although not examined in all models, the perinatal/postnatal increase in foetal ODC activity may be a compensatory response to an initial loss of ODC activity, as the organism attempted to overcome the alcohol-induced growth suppression (Shibley et al., 1995).

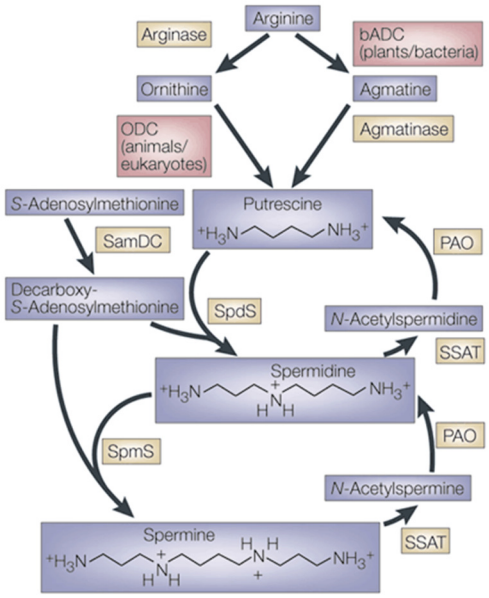

Figure 51: Biosynthesis and catabolism of polyamines. Arginine is converted by the enzyme arginase to ornithine, which in turn is converted by ornithine decarboxylase (ODC) to putrescine during polyamine biosynthesis (Coffino, 2001).

4.5.2. Embryonic gene dysregulation may increase the risk of childhood cancers associated with FASD

In addition to their role in foetal development pathologies, all of the genes (barring *Vwf*) that were identified in our overlap analysis also appear to be associated with the aetiology of various cancers (Table 18). Furthermore, these genes were aberrantly upregulated in embryonic tissues.

The overlap analysis conducted in our current study demonstrated that *Igf1r* was significantly upregulated in the embryonic brain and liver (p=0.0497 and p=0.0084, respectively) with a corresponding significant decrease in DNA methylation (12.35%) in

embryos sired by alcohol exposed males. The insulin-like growth factor-1 receptor (IGF1R) protein is widely expressed across a variety of foetal and postnatal tissues. The binding of IGF1 to IGF1R activates the receptor, where it promotes cell differentiation and proliferation (as reviewed in Zhang, 2012). Several studies have shown an association between altered concentrations of insulin-like growth factor-1 (IGF1) and IUGR (Verkauskiene et al., 2007). Mutations in *IGF1R* have been associated with low birth weight (Kawashima et al., 2012) and intrauterine and postnatal growth restriction in mice (Abuzzahab et al., 2003; Kruis et al., 2010) and humans (Choi et al., 2011; Walenkamp et al., 2006). In addition to growth restriction phenotypes, *Igf1r* mutations have been associated with microcephaly and speech deficits (Abuzzahab et al., 2003), mental retardation (Kawashima et al., 2012), insulin resistance (Walenkamp et al., 2006), decreased cell proliferation (Inagaki et al., 2007), developmental delay (Wallborn et al., 2010), major organ malformation (Veenma et al., 2010), and clinodactyly (curvature of the fifth fingers) (Kruis et al., 2010).

Igf1 and *Igf2* and their corresponding receptors are ubiquitously expressed in neurons and glia (Joseph D'Ercole and Ye, 2008) throughout the brain. The highest levels of Igf1/Igf1r and Igf2/Igf2r are found in the hypothalamus, temporal lobe and cerebellum. These regions of the brain are also the primary targets of alcohol-mediated neurotoxicity. siRNA knock-down of brain insulin or insulin-like growth factor receptors has been shown to cause developmental cerebellar abnormalities (de la Monte et al., 2011), where siRNA inhibition of brain insulin or insulin-like growth factor receptors causes developmental cerebellar abnormalities. These authors noted that the siRNA-mediated structural and functional abnormalities that they observed in the cerebella of rat pups, resembled those of FASD, and concluded that the abnormalities in FASD brains may be mediated by impairments in insulin/Igf signalling. In support of this, ethanol has been shown to impair Igf binding to Igf receptors in postnatal rat pup cerebella (de la Monte et al., 2011), as well as brain insulin/Igf

signalling in adolescent rats subjected to binge alcohol exposures during development (Ewenczyk et al., 2012). These findings are contrary to that of our current study, where increased *Igf1r* expression was observed in the brains (and livers) of embryos sired by ethanol treated sires (associated with decreased DNA methylation at this gene), which was associated with the growth restriction (reduced brain weight) phenotype observed in male embryos. Thus, *Igf1r* overexpression is unlikely to contribute to the observed growth restriction observed in the male embryonic brain.

IGF1R overexpression in humans has been associated with several cancers, including synovial sarcoma (Xie et al., 1999), breast cancers (Almeida et al., 1994), and rhabdomyosarcomas (Bridge et al., 2002). In a study by Almeida et al (Almeida et al., 2008), *IGF1R* overexpression was observed in 65% of children, and 13% of adults with adrenocortical tumours. *IGF1R* is also known to drive growth and metastasis in neuroblastomas. *IGF1R* overexpression have been shown to enhance neuroblastoma tumourigenesis (Singleton et al., 1996), while in mice, repression of *Igf1r* induces the regression of tumour formation in neuroblastoma cell cultures (Liu et al., 1998).

In our current study, *Bcl2l1* was enriched in our CAM model sperm DNA methylation GO analysis within BPs that included sexual reproduction, spermatogenesis and regulation of cell proliferation. We further observed a decrease in DNA methylation at *Bcl2l1* (2.6%, p=0.0119), which was associated with a concomitant increase in gene expression in embryonic the embryonic liver (p=0.0024), but not in the brain (Table 17). The Bcl2 family regulates apoptosis, where Bcl2l1 is an anti-apoptotic protein (Hagenbuchner et al., 2010). An increase of apoptosis-preventing members of the Bcl2 protein family may be able to protect a cell against death under various conditions, including exposure to alcohol (Heaton et al., 1999). Mooney and Miller (Mooney and Miller, 2001) demonstrated that prenatal alcohol exposure reduced *Bcl2* expression in the prenatal rat cortex, which is associated with

apoptosis. Several studies have indicated that alcohol abuse in men can cause impaired testosterone production and shrinkage of the testes (i.e. testicular atrophy) (Adler, 1992). Testicular atrophy also appears to be common among alcoholics, occurring in up to 75 percent of men with advanced alcoholic cirrhosis (Lloyd and Williams, 1948). Subsequent studies have confirmed alcohol's deleterious effects on the testosterone-producing Leydig cells, the Sertoli cells, where alcohol has been shown to induce apoptosis in TM3 mouse Leydig cells via bax-dependent caspase-3 activation (Jang et al., 2002). Kumar et al (2006) observed that procine foetal fibroblasts exposed to the DNA methylation-inhibiting agent 5-azaC showed significant global decreases in DNA methylation, which was associated with decreased expression of *Bcl2l1*, and increased apoptosis. Contrary to this observation, Hanada et al (Hanada et al., 1993) noted 1.7 – 25 fold higher protein levels in peripheral blood lymphocytes in patients with B-cell chronic lymphomatic leukaemia, which was associated with hypomethylation of *Bcl2*. In support of this finding, increased *Bcl2l1* has been associated with prostate cancer development (Zeng et al., 2009). As DNA hypomethylation is generally associated with increased gene expression, in conjunction with the fact that the Kumar et al (2006) quantified DNA methylation at a global level (as opposed to the gene-specific level), it is more likely that *Bcl2* hypomethylation is associated with increased gene expression and protection from apoptosis (thereby enhancing cancer development). Conversely, reduced *Bcl2l1* expression is associated with apoptosis.

Together, these studies suggest that alcohol exposure increases DNA methylation at *Bcl2* which consequently decreases *Bcl2* expression, which results in apoptosis. Apoptosis of this nature might explain the reduced embryonic liver (and brain) weight observed in our current study. However, while an apoptosis-mediated mechanism may be causal to the reduced tissue weights observed, this is not likely attributable to *Bcl2l1* dysregulation. The findings from our current study suggest that paternal preconception alcohol exposure reduces sperm

DNA methylation at *Bcl2l1*, which is associated with increased expression in the embryonic brain and liver.

Further to the above, our study observed significant decreases in sperm DNA methylation at *Ccnd3* (22.27%) which were associated with increased expression in both the embryonic brain and liver (p=0.0465 and p=0.0499, respectively). Mutations in, and consequent dysregulation of, *Ccnd3* is associated with ocular adnexal lymphoma, hepatocellular lymphoma, acute myeloid leukaemia (Smith et al., 2005), T cell acute lymphoblastic leukaemia. It has also been shown that overexpression of *Ccnd3* is associated with T cell acute lymphoblastic leukaemia (Sawai et al., 2012), while Gundogan et al (2013) showed significant downregulation of *Ccnd3* in rat cerebellum following prenatal alcohol exposure (FC=-1.85, p=0.02811). *Igf1r* dysregulation has also been shown to be associated with cancer development. Silencing of *IGF1R* gene has been associated with human prostate cancer (Rochester et al., 2005), while *IGF1R* overexpression has been associated with multiple myeloma in humans (Chng et al., 2006), and is upregulated in primary prostate cancer and persists in metastatic disease (Hellawell et al., 2002). Similarly, *ODC1* overexpression has been associated with prostate cancer (Symes et al., 2013), and plays a role in the development of neuroblastomas (Hogarty et al., 2008), while *Depdc1b* upregulation has been shown to be involved in bladder carcinogenesis (Kanehira et al., 2007) and aggressiveness in breast cancer (Marotta, 2013).

As mentioned previously, FASD has been associated with various cancers in children (Burd et al., 2014), which include blastomas (neuro-, hepato-, and nephroblastoma), Hodgkin disease, leukaemia and rhabdomyosarcoma. Additionally, several studies have shown that chronic parental alcohol consumption is associated with childhood cancers, including acute myeloid leukaemia (Severson et al., 1993; Shu et al., 1996), and acute nonlymphocytic leukaemia (van Duijn et al., 1994). Maternal alcohol consumption prior to conception and during

pregnancy has also been associated with an increased risk of childhood leukaemia (McArthur, 2008). Using a mouse model, Murugan et al (2013) demonstrated that in utero alcohol exposure increases susceptibility to prostate tumourigenesis in rat offspring. Our current study demonstrated overexpression of *Igf1r* and *Ccnd3* in the brain and liver, and overexpression of *Bcl2l1* in the liver, of embryos sired by ethanol exposed males, which are associated with cancers that include neuroblastomas, leukaemias, and rhabdomyosarcomas. These findings may therefore be indicative of the early onset of cancers related to FASD, but mediated paternally following chronic alcohol exposure prior to conception.

Together, preconception and prenatal alcohol exposure have been associated with oncogenesis in subsequent offspring. Dysregulation of *Depdc1b*, *Ccnd3*, and *Igf1r* expression has been associated with similar cancers. Our study demonstrated that paternal preconception alcohol exposure is associated with changes in the expression of these genes in sired offspring. Thus, paternal preconception alcohol exposure may be associated with cancer development in subsequent generations.

Overall, our findings may suggest that the observed decrease in *Odc1* expression observed in the embryonic brain, may account for the observed decrease in brain weight observed in male embryos.

4.6. Alcohol-induced sperm DNA methylation changes dysregulate embryonic gene expression, which may be associated with phenotypes related to FASD, and indicative of paternal transmission of epimutations

In our current study, we observed significant upregulation of *Igf1r* and significant downregulation of *Odc1* expression in the brains of embryos sired by alcohol exposed males. These findings were associated with significant decreases in DNA methylation at these regions in the sperm of alcohol exposed male mice. While a significant decrease in DNA methylation at *Igf1r* might be expected to be associated with the observed increase in *Igf1r* expression, the significant decrease in DNA methylation at *Odc1* would have been expected to be associated with increased *Odc1* expression (which was observed to the contrary). It is therefore possible that the dysregulation of *Odc1* expression is not related to DNA methylation levels at this gene, but as a consequence of the disruption of an alternative epigenetic mechanism not investigated in the current study. Our current study therefore provides empirical evidence (albeit not validated) for the upregulation and downregulation of *Igf1r* and *Odc1*, respectively, as a consequence of preconception paternal alcohol exposure. Further to this, we also observed a significant decrease in embryonic brain and liver weight in male offspring.

4.7. Shortcomings of the current study

Although our study is the first of its kind to extensively investigate the effect of alcohol exposure on the sperm methylome and the transcriptome in tissues of sired offspring, there are several shortcomings and future directions. Firstly, bisulfite conversion and subsequent quantitative bisulfite sequencing lacks the ability to discriminate between 5mC and 5hmC (Kobayashi et al., 2012). This is important to note, as these epigenetic marks appear to have opposite effects on gene regulation. While 5mC is associated with gene repression, 5hmC is associated with an active state (Resendiz et al., 2013). The inability to discriminate between these two epigenetic marks it difficult to draw sound conclusions about observed associations of DNA methylation with other epigenetic mechanisms and gene regulation. Secondly, sucrose exposure in our control mice may in fact also elicit epigenetic changes in sperm DNA methylation of exposed males. This would compromise the assumption that these mice contain "normal"/reference DNA methylation levels. Thirdly, our gene expression analysis did not disaggregate data according to sex. This may be an important analysis, as we only observed growth restriction phenotypes in male embryos. Furthermore, we did not validate RRBS DNA methylation findings with quantitative pyrosequencing, or gene expression data with qRT-PCR. Lastly, we did not study DNA methylation in E16.5 tissues. Because of this, we made the assumption that changes in gene expression in offspring tissues were due to an underlying DNA methylation epimutation, which was inherited from alcohol-exposed sires.

4.8. Concluding remarks

Our previous research (Knezovich and Ramsay, 2012) investigated the effect of chronic (3g/kg/day) alcohol exposure on paternal ICR DNA methylation in the sperm of male mice and in their offspring. It was observed that a significant reduction in DNA methylation was present at only CpG site 7 in the *H19* ICR CTCF2 binding site in the sperm of alcohol exposed males. We further observed significant hypomethylation of the CTCF1 and CTCF2 binding sites in somatic DNA of offspring sired by ethanol-treated males, which was significantly correlated with reduced postnatal weight. More recently, Liang et al (2014) observed that preconception alcohol exposure (3.3 g/kg) of male mice affected DNA methylation at *H19* and *Peg3* (both imprinted genes) in the sperm of exposed mice, and that of *Peg3* (CpG 7 and CpG 11) and *Snrpn* in the cerebral cortices of sired offspring. These authors also assessed neonatal offspring development, and observed delayed cognitive performance and increased anxiety and depression. They concluded that chronic paternal ethanol exposure can affect the methylation of imprinted genes in sire sperm, which may be passed on to offspring, giving rise to mental deficits. However, both of these studies did not investigate whether sperm DNA methylation changes associated with preconception alcohol exposure elicited changes in gene expression in offspring, which may support an epigenetic mechanism and consequently account for the observed phenotypes.

In our present study, we observed significant DNA methylation changes in the mature sperm genome of mice chronically exposed to alcohol (3g/kg/day) prior to conception. We observed 79 regions that showed significant differential methylation in alcohol exposed males, which were common to both mouse models. The direction of change in DNA methylation was in both directions (hypo- and hypermethylated compared to controls). These findings demonstrate that chronic alcohol exposure has the ability to both increase

and decrease sperm DNA methylation. Furthermore, these findings suggest that alcohol-induced sperm epimutations, or at least a subset thereof, are not corrected during spermatogenesis and sperm maturation. These DNA methylation changes were associated with CpG islands at low methylation regions, which are regions of developmental importance. Interestingly, significant DMRs were not observed at imprinting control regions, which are generally either highly methylated (hypermethylated) or devoid of methylation (hypomethylated), and may suggest that these regions are less sensitive or more resilient to the effects of alcohol. GO analysis of the genes associated with significantly differentially methylated sperm DNA regions in the SA and CAM models were associated with common biological processes related to neuronal development, spermatogenesis, regulation of cell proliferation and mobility, and heart development. All of these processes are affected as a consequence of prenatal alcohol exposure, as reflected in the phenotypes associated with FASD.

In our current study, we observed significant changes in the expression of several genes in tissues of E16.5 embryos sired by ethanol exposed males. We observed significant dysregulation of 145 genes across the three tissues investigated (brain, liver, and placenta). Both upregulation (over expression) and downregulation (under expression) of genes was observed, which suggests that epigenetic mechanisms regulating these genes were disrupted as a consequence of paternal alcohol exposure. GO analysis revealed enrichment for biological processes related to skeletal malformations, immune dysfunction and apoptosis, particularly in the brain. These processes are related to foetal alcohol spectrum disorders, and suggest that a similar phenotype may be prevalent in embryos sired by ethanol exposed males.

An overlap analysis of sperm DNA methylation changes and embryonic expression changes revealed that a subset of genes that were dysregulated in embryonic tissues also

demonstrated significantly differential DNA methylated in the sperm of males chronically exposed to alcohol prior to conception. These genes included *Depdc1b*, *Vwf*, *Odc1*, *Ccnd3*, and *Igf1r*. *Vwf*, *Ccnd3* and *Igf1r* demonstrated significant decreases in sperm DNA methylation, which were associated with increases in embryonic gene expression. As described by Guerrero-Bosagna and Skinner (2014), if the mature P0 (parental) sperm epigenome is compromised prior to conception as a consequence of an environmental insult, it is probable that the epimutation will be transmitted to the F1 (first filial) generation. The aberrant paternal epigenome will be mitotically inherited through cellular divisions during embryonic develop, and give rise to the cellular/tissue lineages that would ultimately form the developed embryo. From this, it may be reasonable to conclude that alcohol-induced epimutations were inherited by sired offspring. These significantly differentially methylated regions were contained within regions of low methylation, which are generally associated with the histone-bound fraction of the sperm genome. As there is evidence to suggest that the histone-bound fraction of the sperm genome is able to escape the second round of epigenetic reprogramming that occurs in the early embryo, it is plausible that the alcohol-induced changes in sperm DNA methylation were transmitted to the embryo, where they were propagated during cellular division and embryonic development. As a consequence, gene expression was dysregulated in embryonic tissues. GO analysis of these overlapping genes revealed an enrichment for regulation of apoptosis and cell proliferation.

In addition to the observed changes in sperm DNA methylation, and gene expression in embryos, a growth restriction phenotype was observed in the liver and brain of male embryos sired by ethanol exposed males. Growth restriction is a hallmark of foetal alcohol syndrome, which particularly affects the brain, and in some cases, the liver. These effects are primarily attributed to apoptosis. In our current study, *Odc1* was significantly downregulated in the embryonic brain. *In utero* alcohol exposure has been shown to decrease ODC activity,

and results in growth suppression of the brain. Thus, *Odc1* dysregulation in our study may be associated with reduced embryonic brain weight. Further to this phenotype, foetal alcohol spectrum disorders are associated with childhood cancers. Increased expression of *Depdc1b*, *Ccnd3*, and *Igf1r* expression (as observed in the current study) has been associated with cancers of a similar nature. These findings may be indicative of early onset cancers in our mouse model, which are paternally transmitted, and associated with foetal alcohol spectrum disorders.

Overall, our study provides strong evidence for paternal transmission of epimutations that are associated with dysregulated gene expression, and a growth restriction phenotype in sired offspring. That is, chronic paternal alcohol exposure prior to conception appears to significantly alter sperm DNA methylation, which may be inherited by sired offspring, where gene expression is altered and a growth-restriction phenotype is observed, affecting organs that are primary targets for phenotypes observed in foetal alcohol syndrome.

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Appendices

Appendix A: Swim-out protocol for the isolation of mature spermatozoa

Six to eight month old male mice were sacrificed and their testicles immediately removed. Mature spermatozoa were collected using the following protocol:

1. Using a sharp pair of scissors, gently separate the epididymis from the testis and vas deferens
2. Once removed, gently squeeze and clamp the epididymis head using a pair of fine forceps to build pressure
3. Make a small in the epididymis head using a fine needle
4. Gently extrude the mature spermatozoa by applying pressure to the clamped forceps
5. Repeatedly collect the extruded spermatozoa with the needle using a spooling technique
6. Repeatedly place the extruded spermatozoa in a small volume (200µl) of preheated (37°C) PBS in a small petri dish
7. Repeat this process for the second testicle/epididymis
8. Once all the mature spermatozoa are extruded, aspirate the 200µl of PBS containing the spermatozoa and place it in a 50ml centrifuge tube containing 50ml of preheated (37°C) PBS
9. Incubate the 50ml solution for 10min at 37°C to allow the mature spermatozoa to swim out into the PBS
10. Spin the tube at 1000rpm for 5min at room temperature
11. Remove the supernatant and place it in a fresh tube
12. Spin the supernatant at 3000rpm for 15min at 4°C
13. Discard the supernatant (leave 5ml)
14. Spin at 3000rpm for 5min at 4°C
15. Discard as much of the supernatant as possible

Place the pellet and remaining PBS in a 1.5ml microfuge tube and store at -20°C.

Appendix B: Amendments to the QIAGEN Supplementary Protocol: Purification of DNA from epithelial cells mixed with sperm cells using the QIAamp® DNA Micro Kit

Sperm DNA was extracted from frozen samples obtained from the swim-out protocol (Appendix A) according to the following protocol:

1. Thaw samples
2. Spin samples at 13 300rpm for 10min to pellet the sperm
3. Carefully remove the supernatant (leaving approximately 50µl PBS)
4. Resuspend the sperm pellet in 400µl Buffer ATL
5. Add 18µl of Proteinase K (10µg/ml)
6. Incubate samples at 600rpm for 1 hour at room temperature
7. To each sample, add:
    1. 200µl Buffer ATL
    2. 10µl Proteinase K
    3. 10µl 1M DTT
8. Incubate samples at 900rpm overnight at 56°C
9. Add 300µl Buffer AL
10. Incubate at 900rpm for 10min at 70°C
11. Add 500µml 100% ethanol
12. Briefly votex and incubate for 3min at room temperature
13. Transfer the entire sample to a MinElute column
14. Continue with Steps 15-21 of the QIAGEN Supplementary Protocol as per the manufacturers stipulations

Appendix C: Reduced Representation Bisulfite Sequencing protocol

Protocol obtained with compliments of Dr Sebastien Smallwood (Gavin Kelsey lab, Babraham Institute, UK).

This is the guideline for 10ng starting material.

MspI digestion.

In a PCR tube add:
- Genomic DNA,
- 0.9µl of MspI,
- 1.8µl of Tango 10X buffer,
- H_2O to 18µl final volume.

Incubate for 3h at 37°C, followed by enzyme heat inactivation at 80°C for 20min.

End Repair / A-Tailing:

In the same PCR tube, directly add:
- 1µl of Klenow Fragment exo-,
- 0.8µl of nucleotide end-repair mix, (1mM dATP; 0.1mM dGTP; 0.1mM dCTP)
- 0.2µl of Tango 10X buffer.

Incubate for 40min at 37°C, followed by enzyme heat inactivation at 75°C for 15min.
Proceed to next step or pause.

Adapter ligation:

In the same PCR tube, directly add:
- 1µl of HC T4 DNA ligase,
- 1µl of 5mC sequencing adapters (0.15µM)
- 0.5µl of ATP (50mM),
- 0.5µl of Tango10X
- 2µl of H_2O.

Incubate overnight at 4°C, followed by enzyme heat inactivation at 65°C for 20min. (**NOTE5**).
Proceed to next step or pause.

Bisulfite modification:

Take 24µl of the previous reaction mix (step 3.4) and use directly the Imprint DNA Modification Kit 2-step modification procedure, with the following modification: once DNA modification mix is added, incubate at 65°C for 90min, 99°C for 2min, 65°C for 30min.

Perform purification steps according to the manufacturer's protocol, and elute DNA in 43-45µl of warm EB buffer.

Proceed to next step or pause.

Amplification step:

To the BS converted DNA, set up a 50µl PCR reaction by adding:
- 41µl of bisulfite treated DNA,
- 1µl of Pfu Turbo Cx Hotstart DNA polymerase
- 5µl of Pfu Turbo Cx Hotstart 10X buffer,
- 1µl of dNTP mix,
- 1µl of primers PE1.0,
- 1µl of primers PE2.0.

Perform PCR cycles (95°C-2min; 95°C-20sec, 65°C-20sec, 72°C-30sec).
16 cycles should be largely ok for 10ng.
(Alternatively perform a PCR test with 1µl to define PCR cycles)

Amplification step:

AMPure XP beads.

Appendix D: Significant DMRs from SA and CAM models

SA model (388 DMRs)

CHR	START	END	SIZE	METHYLATION	ETOH-1	ETOH-2	ETOH-3	CTRL-1	CTRL-2	CTRL-3	ABS_aveDIFF	P.Value	GENE	DIST	GenBank
chr1	5160001	5161300	1300	HYPER	82.9	84.1	66.7	72.8	73.4	71.3	11.0	<0.01	Atp6v1h	7371	NM_133826
chr1	29951601	29952500	900	HYPER	74	75.2	76	57.7	57.3	62.1	15.6	<0.01	.	.	NM_001081080
chr1	50869001	50869900	900	HYPER	47.2	50	54.2	38.2	36.4	31.8	13.1	<0.01	.	.	NM_019790
chr1	66616001	66616900	900	HYPER	62.6	62.9	64.9	60.4	42.9	54.6	10.1	<0.01	Unc80	0	NM_175510
chr1	74759601	74760500	900	HYPER	26.7	26.7	.	16.7	.	.	10.0	<0.01	Cyp27a1	0	NM_024264
chr1	116172801	116173700	900	HYPER	84.6	84.7	81.8	79	78	72.9	8.0	<0.01	.	.	NM_001077425
chr1	172543001	172543900	900	HYPER	86.6	88	87.9	79.8	73.7	81.4	9.0	<0.01	Nos1ap	-23021	NM_001109985
chr1	175092001	175092900	900	HYPER	100	98.7	.	.	87.5	.	11.9	<0.01	Olfr1406	20672	NM_146763
chr2	28468001	28468900	900	HYPER	55.5	57.6	48.2	40.3	51	44.5	11.3	<0.01	Gfi1b	0	NM_001160406
chr2	28493201	28494300	1100	HYPER	25	21.9	7.4	19.9	9	13.9	9.2	<0.01	Tsc1	-2462	NM_022887
chr2	44210401	44211300	900	HYPER	61.1	56.5	.	46.7	39.3	33.3	19.0	<0.01	Arhgap15	0	NM_153820
chr2	72252201	72253100	900	HYPER	74.8	80.2	58	63.9	62.5	66.9	13.1	<0.01	B230120H23Rik	0	NM_023057
chr2	73585801	73586500	700	HYPER	100	100	.	.	90	.	10.0	<0.01	Chn1	0	NM_001113246
chr2	80443601	80444500	900	HYPER	38.3	39.8	45.3	29.5	22.8	33.6	10.4	<0.01	Dusp19	-12870	NM_024438
chr2	82422401	82423300	900	HYPER	71.2	72.6	52.4	58.8	54.3	66.1	12.2	<0.01	.	.	NM_175513
chr2	82714401	82715300	900	HYPER	91.7	94.4	93.3	80	.	.	13.1	<0.01	.	.	NM_175513
chr2	129069201	129070300	1100	HYPER	56.8	56.8	54.8	47.6	46.7	49.1	9.0	<0.01	A730036I17Rik	7859	NR_045838
chr2	159974201	159975100	900	HYPER	93.6	92.3	100	85.6	85.1	76.5	10.6	<0.01	.	.	NR_040499
chr2	168236201	168237100	900	HYPER	43.2	38.8	58	20.5	33.2	31.8	12.5	<0.01	Nfatc2	64809	NM_010899
chr2	170509201	170510100	900	HYPER	100	100	.	91.7	81.7	83.3	14.4	<0.01	Dok5	-47206	NM_001163686

	17222020	17222130	110												NM_00103353
chr2	1	0	0	HYPER	35.1	35.1	21.9	23.2	27.5	28.3	8.8	<0.01	Cass4	0	8
	17353780	17353890	110										1700010B08Ri		
chr2	1	0	0	HYPER	83.4	84	.	75.2	67.8	71.2	12.3	<0.01	k	-6015	NM_029308
chr3	9520201	9521100	900	HYPER	84.6	82.8	86	70.9	75.6	71.3	11.1	<0.01	Zfp704	0	NM_133218
chr3	27150801	27151700	900	HYPER	41.4	48.3	39.5	33.6	28.6	37.5	11.6	<0.01	Nceh1	6968	NM_178772
			110											-	
chr3	65259001	65260100	0	HYPER	40.8	44	19	33.8	31.2	28.2	11.3	<0.01	Ssr3	62526	NM_026155
			130												
chr3	72813401	72814700	0	HYPER	95.8	97.9	.	87.5	79.6	88.2	11.8	<0.01	Slitrk3	37346	NM_198864
chr3	89136201	89137100	900	HYPER	52.7	52.8	49.5	41.9	45.7	40	10.2	<0.01	Ef.4	214	NM_007910
															NM_00103773
chr3	95237801	95238500	700	HYPER	18.3	19.9	42.9	8.9	11.8	4.2	10.8	<0.01	Arnt	0	7
	12980620	12980710												-	
chr3	1	0	900	HYPER	63.3	60.1	27.8	57.8	42.1	46.9	12.8	<0.01	Sec24b	42376	NM_207209
	13046400	13046490												-	NM_00119935
chr3	1	0	900	HYPER	37.9	35.4	14.8	23.2	21.7	28	12.4	<0.01	Rpl34-ps1	30754	0
	13227420	13227510												-	NM_00127043
chr3	1	0	900	HYPER	62.6	60.8	54.5	40.2	59.4	53.7	10.6	<0.01	Gm5549	18054	0
	13279480	13279550													
chr3	1	0	700	HYPER	100	100	.	80.6	88.4	91.7	13.1	<0.01	Ints12	20852	NM_027927
	13434740	13434810													
chr3	1	0	700	HYPER	75.2	75.2	83.3	56.5	75.2	62.5	10.5	<0.01	.	.	NM_021382
chr4	8307801	8308500	700	HYPER	40.3	38.5	30	29.6	23.7	25.8	13.0	<0.01	.	.	NM_007592
chr4	11632801	11633700	900	HYPER	100	100	.	92.3	86.7	90.5	10.2	<0.01	Gem	0	NM_010276
														-	
chr4	26297001	26297900	900	HYPER	15.2	18.2	13.3	2.6	5.9	.	12.5	<0.01	Manea	23202	NM_172865
			170												
chr4	85409601	85411300	0	HYPER	49.2	38.8	36	31	28.7	20.9	17.1	<0.01	.	.	NM_029967
	13050140	13050210													
chr4	1	0	700	HYPER	78.6	75	70.1	70.8	61.5	63.7	11.5	<0.01	Matn1	0	NM_010769
	13077420	13077510													
chr4	1	0	900	HYPER	38.3	34.3	33.8	9.1	23.6	22	18.1	<0.01	.	.	NM_010769
	13422180	13422270													
chr4	1	0	900	HYPER	64.1	56.5	80	48	44	35	18.0	<0.01	Man1c1	0	NM_207237
	13823200	13823290													
chr4	1	0	900	HYPER	71.4	70.6	50	52.2	51.7	56.6	17.5	<0.01	Ubxn10	41540	NM_178671
	13959420	13959550	130												
chr4	1	0	0	HYPER	29.1	27.8	25.7	17.4	17.1	9.2	13.9	<0.01	Igsf21	0	NM_198610
	15219760	15219850													
chr4	1	0	900	HYPER	80	80.3	.	63	82.7	66.7	9.4	<0.01	.	.	NR_033138
	15248060	15248130													NM_00109929
chr4	1	0	700	HYPER	96.3	100	.	83.3	.	88.9	12.1	<0.01	.	.	9
chr5	25173401	25174300	900	HYPER	36.3	41	32.4	29	25.8	22	13.1	<0.01	Xrcc2	21333	NM_020570

chr5	71039401	71040100	700	HYPER	96.9	94.9	84.3	84.4	82.5	96.8	8.0	<0.01	.	.	NM_010252
chr5	75525401	75526100	700	HYPER	100	100	.	85.7	94.9	80	13.1	<0.01	Gm19583	0	NR_045792
chr5	82728601	82729300	700	HYPER	82	80	.	55.6	60	.	23.2	<0.01	.	.	NR_035415
chr5	87665801	87666700	900	HYPER	82	78.1	84.2	65.8	61.2	79.5	11.2	<0.01	Ugt2b37	2816	NM_053215
chr5	102075201	102076100	900	HYPER	24.4	24	28.7	11.3	10	20.5	10.3	<0.01	Nkx6-1	12115	NM_144955
chr5	103797601	103798500	900	HYPER	41.7	41.2	29.6	24.3	25	37.9	12.4	<0.01	Ptpn13	-55710	NM_011204
chr5	104008401	104009300	900	HYPER	68.7	62.4	59.8	57.9	46	54.2	12.9	<0.01	Ptpn13	0	NM_011204
chr5	105343801	105344700	900	HYPER	90	87.6	90.1	74.9	71.7	77	14.3	<0.01	Abcg3	19375	NM_030239
chr5	114264801	114265700	900	HYPER	27.9	28.7	10.2	22	12.7	22.2	9.3	<0.01	Selplg	2106	NM_009151
chr5	115046601	115047901	1300	HYPER	37.9	37.5	36.7	16.5	35.4	32.5	9.6	<0.01	BC057022	0	NM_001004180
chr5	115950201	115951101	900	HYPER	61.4	66.1	61.9	50.1	52.3	58.1	10.3	<0.01	Pxn	-5610	NM_011223
chr5	120372201	120373101	900	HYPER	46.4	50.7	51.4	32.4	34.9	40.2	12.7	<0.01	Tbx5	36974	NM_011537
chr5	121664801	121665701	900	HYPER	47.6	44.4	60	39.4	34.7	25.5	12.8	<0.01	Gm15800	-4527	NM_181421
chr5	129476801	129477701	900	HYPER	30	31.2	.	30.2	16.6	6.8	12.7	<0.01	Stx2	12735	NM_007941
chr5	134368201	134369301	1100	HYPER	31	37.8	34.8	25.8	23.4	18	12.0	<0.01	.	.	NM_030719
chr5	135456801	135457901	1100	HYPER	24.7	25.4	25.6	19	14.7	15.6	8.6	<0.01	Cldn3	-4183	NM_009902
chr5	148618001	148619101	1100	HYPER	89.4	91.4	88.9	77	83.6	79.8	10.3	<0.01	Pomp	-53103	NM_025624
chr5	148838801	148839901	1100	HYPER	29	27.5	11	20.2	19.9	13	10.6	<0.01	Mtus2	0	NM_029920
chr5	151758201	151758901	700	HYPER	38.2	35.5	30.4	26.9	21.4	20.1	14.1	<0.01	Kl	0	NM_013823
chr5	151784801	151785501	700	HYPER	46.7	43.7	.	35.7	39.4	29.6	10.3	<0.01	Kl	0	NM_013823
chr6	34958601	34959501	900	HYPER	100	100	100	.	85.7	.	14.3	<0.01	Cnot4	12564	NM_001164412
chr6	55346401	55347301	900	HYPER	95.2	100	.	.	83.3	.	14.3	<0.01	6430584L05	0	NR_046179
chr6	68649201	68650101	900	HYPER	62.6	62.7	69.7	59.7	57.1	43.7	9.2	<0.01	.	.	NR_004434
chr6	86183601	86184501	900	HYPER	58.3	62.6	55.6	46.1	46.3	52.1	12.3	<0.01	Tgfa	0	NM_031199
chr6	87445401	87446501	1100	HYPER	100	100	.	100	86.2	.	6.9	<0.01	Arhgap25	0	NM_175476

chr6	88110801	88111900	1100	HYPER	51.6	51.5	45	49.2	30.3	41	11.4	<0.01	Gata2	-36757	NM_008090
chr6	96065201	96066100	900	HYPER	25	23.8	.	13.9	25	.	5.0	<0.01	Fam19a1	0	NM_182808
chr6	104871601	104872500	900	HYPER	49	52	39.1	47.6	39.7	33.6	10.2	<0.01	Cntn6	58202	NM_017383
chr6	128556001	128556900	900	HYPER	74.2	74.4	75.7	65.5	66.5	63.8	9.0	<0.01	Klrb1a	2344	NM_001159902
chr6	141055201	141056100	900	HYPER	61.2	58.3	45.9	55.3	48.5	40.3	11.7	<0.01	.	.	NM_018779
chr6	141652801	141653700	900	HYPER	97.9	100	100	85.1	92.6	89.2	10.0	<0.01	Slco1b2	17645	NM_020495
chr7	15554801	15555900	1100	HYPER	74.2	74.2	72.7	63.9	63.1	76.2	6.5	<0.01	.	.	NM_001038676
chr7	16571801	16572700	900	HYPER	37.5	33.3	.	10	11.1	25	20.0	<0.01	Gltscr1	0	NM_001081418
chr7	25445801	25447300	1500	HYPER	72.4	70.2	72	60.4	63.3	61.7	9.5	<0.01	Gm4598	0	NR_030681
chr7	33276401	33277100	700	HYPER	71.4	74.3	77.3	69.4	57.7	58.9	10.9	<0.01	Scgb1b29	48538	NM_001256066
chr7	36323401	36324300	900	HYPER	86.7	86.7	.	.	.	75	11.7	<0.01	Tdrd12	-638	NM_028034
chr7	51868601	51870100	1500	HYPER	38.9	39.8	41.1	30.2	30.9	29.2	9.3	<0.01	Myh14	0	NM_001271538
chr7	62664601	62665500	900	HYPER	95.5	100	.	84.4	87.5	78.3	14.4	<0.01	.	.	NM_178705
chr7	65378801	65379700	900	HYPER	95.8	96.9	.	.	85.7	.	10.7	<0.01	.	.	NM_001038701
chr7	69791001	69791700	700	HYPER	86.1	88	83.3	75	75	79.3	10.6	<0.01	.	.	NM_013788
chr7	105834001	105834900	900	HYPER	44.4	43.5	0	39.8	32.2	30.7	9.7	<0.01	Prkrir	-16972	NM_028410
chr7	147361801	147362700	900	HYPER	76.8	82	81	58.1	44.4	55.9	26.6	<0.01	Olfr523	0	NM_146518
chr7	150689601	150690500	900	HYPER	78.9	79.2	15.4	66	67	70.8	11.1	<0.01	Phlda2	-1172	NM_009434
chr8	4408001	4408700	700	HYPER	93.6	93	78.3	76.6	87	91.2	8.4	<0.01	Ccl25	47981	NM_009138
chr8	4800401	4801100	700	HYPER	100	100	.	86.7	.	.	13.3	<0.01	Shcbp1	-20867	NM_011369
chr8	18738401	18739500	1100	HYPER	68.3	62.3	61.1	48.1	53	55.6	13.1	<0.01	Angpt2	0	NM_007426
chr8	42142601	42143500	900	HYPER	83.3	88.2	.	74.2	70.7	62.9	16.5	<0.01	Mtus1	0	NM_001005863
chr8	46513601	46514300	700	HYPER	88.6	89	90	87.5	81.3	67.5	10.0	<0.01	Tlr3	-17708	NM_126166
chr8	53988801	53989700	900	HYPER	80.9	79.2	75	73.8	72.1	67.2	9.0	<0.01	.	.	NM_001005847
chr8	67096001	67096900	900	HYPER	85.4	87.7	93.8	74.8	89.8	66.2	9.6	<0.01	Cpe	0	NM_013494

chr8	93710601	93711700	1100	HYPER	83.3	82.9	78.6	77.2	72.6	69.8	9.9	<0.01	Rpgrip1l	29228	NM_173431
chr8	100275801	100276500	700	HYPER	69.4	67.1	.	48.6	69.8	52.5	11.3	<0.01	.	.	NM_001039154
chr8	112048801	112049700	900	HYPER	70.6	71.2	.	54.8	53.6	47.6	18.9	<0.01	Pmfbp1	0	NM_019938
chr8	116100201	116100901	700	HYPER	66.7	63.4	48.6	50	52.4	61.1	10.6	<0.01	Mon1b	-58585	NM_001048143
chr9	4260001	4261100	1100	HYPER	33.3	37.9	36.5	23.8	23.3	28.1	10.5	<0.01	Aasdhppt	33692	NM_026276
chr9	18821601	18822300	700	HYPER	88.9	89.6	71.4	61.9	82.3	77.8	15.3	<0.01	Olfr835	-17268	NM_001012266
chr9	45362801	45363700	900	HYPER	69.2	67	50	64.5	66	48.2	8.5	<0.01	Dscaml1	0	NM_001081270
chr9	46169401	46170500	1100	HYPER	100	100	96.4	93	86	97.6	7.8	<0.01	4931429L15Rik	-41332	NM_183104
chr9	48933801	48934700	900	HYPER	39.1	35.5	27.7	19.8	20.5	26.4	15.1	<0.01	Tmprss5	8108	NM_030709
chr9	72704201	72705100	900	HYPER	54.3	50.1	64.4	41.4	39.4	41.8	11.3	<0.01	Prtg	0	NM_175485
chr9	101988001	101989101	1100	HYPER	89.7	84.8	.	65	90.2	82.9	7.9	<0.01	Ephb1	0	NM_001168296
chr9	107095801	107096501	700	HYPER	27.3	33.3	.	16.7	16.8	19.1	12.8	<0.01	Dock3	0	NM_153413
chr9	115215201	115216101	900	HYPER	83.9	83.1	.	69.4	57.5	.	20.1	<0.01	Stt3b	0	NM_024222
chr10	6389401	6390300	900	HYPER	89.2	93.2	98.6	86.2	76.2	83	9.4	<0.01	Plekhg1	0	NM_001033253
chr10	18150401	18151300	900	HYPER	48.8	70.3	37.8	45.6	38.6	37.9	18.9	<0.01	Nhsl1	0	NM_173390
chr10	19924801	19925500	700	HYPER	26.3	31.4	24.4	10.8	10.4	15.4	16.7	<0.01	Mtap7	0	NM_001198635
chr10	22378201	22379100	900	HYPER	22.2	25.2	.	14.3	9.5	13	11.4	<0.01	Slc2a12	0	NM_178934
chr10	41682801	41683900	1100	HYPER	80.5	77.8	61.6	74.8	67	64	10.6	<0.01	Armc2	0	NM_001034858
chr10	58121401	58122300	900	HYPER	87.9	80.4	84.2	84.6	62.4	68.9	12.2	<0.01	Edar	0	NM_010100
chr10	59030001	59030700	700	HYPER	83.4	86.7	93.2	80.2	80	61.3	11.2	<0.01	Ccdc109a	0	NM_001033259
chr10	66181801	66182700	900	HYPER	29.7	27.6	30.3	19.2	12.5	21.9	10.8	<0.01	.	.	NM_001204915
chr10	75035801	75037100	1300	HYPER	100	100	100	98	89.2	100	4.3	<0.01	Ggt1	0	NM_008116
chr10	80779401	80780500	1100	HYPER	78.9	82.1	.	64.7	69.6	56.4	16.9	<0.01	Pip5k1c	0	NM_001146687

chr10	105076801	105077700	900	HYPER	74.1	66.4	59.1	48.2	61.2	59.3	14.0	<0.01	Gm15663	55875	NR_038032
chr10	120752201	120752900	700	HYPER	72.2	71.4	.	27.8	26.7	.	44.6	<0.01	Tbc1d30	-3956	NM_029057
chr11	4208201	4209300	1100	HYPER	56.3	54.4	38.7	42	44.5	50	9.8	<0.01	Lif	35684	NM_001039537
chr11	15092601	15093500	900	HYPER	91.7	87.8	.	74.3	77.6	86.6	10.3	<0.01	.	.	NM_001164166
chr11	24462001	24463100	1100	HYPER	92.8	86.3	57.1	82.1	76.2	74.7	11.9	<0.01	4933430M04Rik	0	NR_045857
chr11	41553201	41553900	700	HYPER	78.2	79.2	86.7	57.1	65	75.3	12.9	<0.01	.	.	NM_177408
chr11	53278201	53279100	900	HYPER	73.7	68.7	45	55.5	64.7	59.7	11.2	<0.01	Shroom1	0	NM_027917
chr11	59170001	59170900	900	HYPER	100	100	.	80	100	91.1	9.6	<0.01	Prss38	15270	NM_001045521
chr11	61126601	61127500	900	HYPER	50.2	52.8	36.4	29.4	45	38.2	14.0	<0.01	Slc47a2	0	NM_001033542
chr11	69179601	69180300	700	HYPER	13.9	12.8	.	11.8	0	0	9.4	<0.01	Chd3	0	NM_146019
chr11	93999001	94000300	1300	HYPER	36.2	37	28.7	25.4	22.8	25.6	12.0	<0.01	Spag9	11605	NM_001199203
chr11	109106001	109106900	900	HYPER	90.7	91.2	95.8	76.8	80.5	77.8	12.6	<0.01	Rgs9	0	NM_011268
chr11	112731001	112732700	1700	HYPER	54.5	55.2	40.9	40.4	49.9	48.8	8.5	<0.01	Sox9	81930	NM_011448
chr12	149448001	149455001	700	HYPER	98.1	100	.	97.2	75	95.8	9.7	<0.01	4930448C13Rik	57694	NR_045960
chr12	200774001	200783001	900	HYPER	51.2	50.4	60	33.9	47.7	38.7	10.7	<0.01	.	.	NR_015521
chr12	462960001	462973001	1300	HYPER	48.4	52.8	59	48.6	39.8	52.2	3.7	<0.01	.	.	NM_144552
chr12	783180001	783189001	900	HYPER	69	71.8	49	63.3	56.8	61.6	9.8	<0.01	Fut8	-20190	NM_001252614
chr12	811034001	811043001	900	HYPER	64.8	76.6	58.8	58.6	54.4	48.9	16.7	<0.01	.	.	NM_007564
chr12	100907401	100908901	1500	HYPER	68.8	68.1	28.6	57.4	61.7	70.1	5.4	<0.01	1700064M15Rik	-41217	NR_045288
chr12	107796601	107797501	900	HYPER	76.8	79.1	91.1	71.4	65.9	64.4	10.7	<0.01	.	.	NR_036593
chr12	108787401	108788901	1500	HYPER	25.8	27	18.8	10.8	15.3	23.6	9.8	<0.01	.	.	NR_045973
chr12	114437601	114438301	700	HYPER	95.8	95.2	.	85.4	83.3	88.9	9.6	<0.01	Tmem121	9868	NM_153776

chr13	3978001	3978700	700	HYPER	91.2	86.1	.	87.5	71.6	67.8	13.0	<0.01	Ucn3	-33406	NM_031250
chr13	14056801	14057900	1100	HYPER	27	25.4	33.4	15.1	18.2	13.2	10.7	<0.01	B3galnt2	0	NM_178640
chr13	39103001	39104300	1300	HYPER	28.2	30.8	11.7	17.2	22.4	11	12.6	<0.01	Slc35b3	-50257	NM_001170431
chr13	48254201	48255100	900	HYPER	100	96	90	82.5	94	85.8	10.6	<0.01	.	.	NM_031166
chr13	52477001	52477700	700	HYPER	93.9	87.5	85.2	84.8	81.5	72	11.3	<0.01	.	.	NM_001024474
chr13	56542001	56542700	700	HYPER	89.8	88.2	88.9	73.6	77	79	12.5	<0.01	Slc25a48	0	NM_177809
chr13	58102401	58103300	900	HYPER	43.3	40.5	.	13.9	15.8	25.6	23.5	<0.01	Klhl3	3017	NM_001195075
chr13	81833201	81834100	900	HYPER	48.6	50.4	53.4	44.8	37.8	30.8	11.7	<0.01	Polr3g	0	NM_001081176
chr13	88899801	88900900	1100	HYPER	73.7	68.6	40	58.9	45.5	44.1	21.7	<0.01	Edil3	-60176	NM_001037987
chr14	14610401	14611300	900	HYPER	15.5	23	16.4	19.8	7.1	8.3	7.5	<0.01	Sntn	94739	NM_177624
chr14	16743601	16745300	1700	HYPER	84.8	76.7	72	60.2	57.3	70.6	18.1	<0.01	.	.	NM_027695
chr14	40325801	40326700	900	HYPER	95.8	95.8	.	87.5	100	100	0.0	<0.01	Nrg3	-39425	NM_001190187
chr14	52624601	52625500	900	HYPER	84	83.9	65.6	76.8	73.3	76.2	8.5	<0.01	Arhgef40	0	NM_001145921
chr14	64951401	64952300	900	HYPER	71.1	70.9	50	57.9	38.1	56.5	20.2	<0.01	Msra	0	NM_001253716
chr14	70033401	70034500	1100	HYPER	91.5	91.9	86.8	82.4	90.3	65.3	12.4	<0.01	Loxl2	0	NM_033325
chr14	75373601	75374300	700	HYPER	96.7	100	.	76.9	86.7	94.4	12.4	<0.01	Gm4278	-592	NR_046078
chr14	116606001	116606700	700	HYPER	31.7	31.6	16.1	21.4	28.1	13.3	10.7	<0.01	Gpc5	0	NM_175500
chr15	6819401	6820300	900	HYPER	44.6	47.6	.	16.7	35.8	34.6	17.1	<0.01	Osmr	0	NM_011019
chr15	25466401	25467300	900	HYPER	13.9	13.1	5	5	4.8	2.4	9.4	<0.01	Gm5468	84228	NR_027376
chr15	81810601	81811500	900	HYPER	31.2	35	.	12.5	16.6	14.3	18.6	<0.01	1700029P11Rik	0	NM_025503
chr15	83673201	83674100	900	HYPER	100	100	.	98.6	100	73.8	9.2	<0.01	Mpped1	0	NM_172610
chr15	85157801	85158700	900	HYPER	72.8	68.3	65.1	63.5	59.4	54.8	11.3	<0.01	Atxn10	-8110	NM_016843

chr16	20907001	20907900	900	HYPER	83.3	83.5	76.1	66.7	75.6	82.7	8.4	<0.01	.	.	NM_009893
chr16	43969001	43969700	700	HYPER	40	33.5	47.3	26.7	22.5	23.7	12.5	<0.01	Zdhhc23	0	NM_001007460
chr16	64365801	64366500	700	HYPER	36.1	27.8	.	16.6	21.6	.	12.9	<0.01	.	.	NM_173861
chr16	77075601	77076500	900	HYPER	100	100	100	100	90.5	100	3.2	<0.01	Usp25	0	NM_013918
chr16	89774401	89775300	900	HYPER	13.5	22	0	10.2	3.9	3.6	11.9	<0.01	Tiam1	12055	NM_001145887
chr17	10911801	10913300	1500	HYPER	67.8	74.2	100	62.8	54.4	58.2	12.5	<0.01	Pacrg	0	NM_027032
chr17	17482601	17483500	900	HYPER	87.6	87.9	75.2	73.8	89.5	70.4	9.8	<0.01	Riok2	-27795	NM_025934
chr17	25727001	25727900	900	HYPER	85.1	90.9	91.4	77.9	82.7	70.5	11.0	<0.01	Lmf1	0	NM_029624
chr17	34726401	34727700	1300	HYPER	62	57.2	43.9	44.4	41.5	44.6	16.1	<0.01	Gpsm3	0	NM_134116
chr17	86331601	86332700	1100	HYPER	39.7	41.7	.	13.3	35.7	.	16.2	<0.01	Srbd1	51304	NM_030133
chr18	3975801	3976700	900	HYPER	75	75	100	56.4	69	63.9	11.9	<0.01	.	.	NM_026092
chr18	7151001	7151700	700	HYPER	73.6	66.6	.	34.5	47.1	58.6	23.4	<0.01	Armc4	0	NM_001081393
chr18	53757201	53758300	1100	HYPER	89.2	84.1	86	77	74.1	61.8	15.7	<0.01	Prdm6	21690	NM_001033281
chr18	65665001	65666100	1100	HYPER	35.5	45.9	55.4	35.5	19.6	26	13.7	<0.01	Malt1	26555	NM_172833
chr18	73595201	73596300	1100	HYPER	60.9	62.8	59.3	56.2	36.3	54.6	12.8	<0.01	.	.	NM_001039214
chr18	83082001	83083100	1100	HYPER	23.6	23.8	.	10.6	0	25	11.8	<0.01	Zfp516	0	NM_183033
chr19	18976201	18977700	1500	HYPER	28.7	28.7	14.7	14.8	25.6	18.5	9.1	<0.01	Trpm6	9200	NM_153417
chr19	38183801	38184700	900	HYPER	98.3	90.5	89.6	78.7	76.8	90.7	12.3	<0.01	O3far1	0	NM_181748
chr19	43926401	43927300	900	HYPER	73.3	71.2	.	56.6	69.8	61	9.8	<0.01	Dnmbp	0	NM_028029
chr19	47347601	47348500	900	HYPER	100	100	100	100	100	40	20.0	<0.01	Sh3pxd2a	0	NM_001164717
chr19	48306001	48306900	900	HYPER	94.8	84.9	100	70.4	83.4	69.3	15.5	<0.01	Sorcs3	0	NM_025696
chrX	23730601	23731500	900	HYPER	90.5	90.7	66.7	82.3	79	81.1	9.8	<0.01	.	.	NM_001034864

chrX	37252801	37253700	900	HYPER	61.4	58.1	53.3	40.3	30.4	51.6	19.0	<0.01	Cypt14	-36020	NM_001191032
chrX	91792401	91793500	1100	HYPER	47.1	45.5	61.9	44.3	31.3	34.8	9.5	<0.01	Maged1	-4988	NM_019791
chrX	96901601	96902500	900	HYPER	100	100	.	.	.	85.7	14.3	<0.01	.	.	NM_001081283
chrX	100958201	100959100	900	HYPER	19	18.1	11.1	8.9	0	13.9	11.0	<0.01	Gm6222	0	NM_001170979
chrX	115258801	115259700	900	HYPER	70.7	54	60	47.5	29.3	53	19.1	<0.01	.	.	NM_001142750
chrX	119580001	119580900	900	HYPER	52.1	55.6	44.8	40.3	38.3	40.6	14.1	<0.01	.p1l3	-69007	NM_138742
chrX	143693601	143694500	900	HYPER	34.6	35.9	43.9	.	17.5	.	17.8	<0.01	Htr2c	61781	NM_008312
chrX	151146801	151147500	700	HYPER	93.9	93.5	78.3	74.3	84.4	74.1	16.1	<0.01	.	.	NM_001037167
chrX	162287601	162288500	900	HYPER	85.4	88.5	77.8	77.5	63.9	77.3	14.1	<0.01	.	.	NM_146238
chr1	40111801	40112900	1100	HYPO	13.6	14.2	11.9	23.4	17.8	27.1	8.9	<0.01	Gm16894	20156	NR_037980
chr1	136186401	136187501	1100	HYPO	22.7	13.6	18.8	30.3	23.7	24.9	8.2	<0.01	Myog	0	NM_031189
chr1	137582601	137583901	1300	HYPO	19.5	21.7	6.8	34	32.1	27.5	10.6	<0.01	Csrp1	-41873	NM_007791
chr1	144512001	144512901	900	HYPO	83.3	83.3	.	92	.	.	8.7	<0.01	.	.	NM_145991
chr1	177985601	177986501	900	HYPO	40.6	41.6	48.9	47.9	49.3	57.1	10.3	<0.01	Pld5	0	NM_001195816
chr2	102306001	102315001	900	HYPO	54	51.7	48.6	75.2	55	67.6	13.1	<0.01	Itih5	52445	NM_172471
chr2	192394001	192403001	1500	HYPO	47.7	41.3	58.3	49	65.8	59.4	13.6	<0.01	Armc3	7531	NM_001081083
chr2	311112001	311127001	1500	HYPO	73.1	72.1	72.1	82.9	87.8	80.8	11.2	<0.01	Ncs1	0	NM_019681
chr2	325020001	325029001	900	HYPO	4.8	5.1	8.1	11.9	7.9	14.1	6.4	<0.01	Eng	0	NM_001146348
chr2	733812001	733823001	1100	HYPO	8.8	8	30	26	18.3	9.3	9.5	<0.01	Wipf1	-13734	NM_153138
chr2	743290001	743301001	1100	HYPO	31.1	38.6	.	56.5	56.8	50.5	19.8	<0.01	Lnp	22793	NM_001110209
chr2	821826001	821835001	900	HYPO	78.5	78.2	86.2	92.1	90.6	79.5	9.1	<0.01	Zfp804a	82566	NM_175513
chr2	105193601	105194701	1100	HYPO	40.8	40.2	36.2	59.9	43.7	47.2	9.8	<0.01	0610012H03Rik	0	NM_001159638
chr2	105213201	105214101	900	HYPO	24.8	22.8	19.8	36.7	29.9	40.5	11.9	<0.01	0610012H03Rik	0	NM_001159638

chr2	10526880 1	10526970 0	900	HYPO	63	60	82.4	82.4	88.9	.	24.2	<0.01	Rcn1	-29325	NM_009037
chr2	13552860 1	13552950 0	900	HYPO	0	2.3	.	32.3	13.5	27.5	23.3	<0.01	Plcb4	-38065	NM_013829
chr2	15557660 1	15557750 0	900	HYPO	18.1	14.7	.	29.5	19.6	29.4	9.8	<0.01	Procr	0	NM_011171
chr2	15797180 1	15797250 0	700	HYPO	0	0	.	12	0	13.8	8.6	<0.01	Tgm2	0	NM_009373
chr2	15905340 1	15905410 0	700	HYPO	53.4	53.8	54.1	63.9	66.4	63.5	11.0	<0.01	.	.	NM_145742
chr2	16753740 1	16753830 0	900	HYPO	83.5	83.9	93.7	98.1	95.8	89.6	10.8	<0.01	A530013C23Rik	14488	NR_015500
chr2	16868640 1	16868710 0	700	HYPO	44.4	58.3	47.6	86.7	71.7	78.8	27.7	<0.01	Zfp64	63760	NM_009564
chr3	10162601	10163500	900	HYPO	30.3	28.5	12.5	42	49	43.5	15.4	<0.01	Pmp2	16350	NM_001030305
chr3	32743401	32744300	900	HYPO	81.7	83	91.2	97.3	95.5	88.1	11.3	<0.01	Usp13	0	NM_001013024
chr3	37498201	37499300	1100	HYPO	27.4	23.3	16.2	44.1	42.2	23.7	11.3	<0.01	Spata5	20184	NM_001163511
chr3	52728801	52729700	900	HYPO	51.3	46.9	64.1	65.3	53.1	61.3	10.8	<0.01	LOC622124	0	NR_040555
chr3	78562201	78563100	900	HYPO	38.3	36.7	44	41.1	51	54.6	11.4	<0.01	.	.	NM_001099624
chr3	88352201	88353100	900	HYPO	47.4	49.6	60.7	56.8	62.1	58.6	10.7	<0.01	Rab25	0	NM_016899
chr3	10572300 1	10572390 0	900	HYPO	27.8	19.7	28	27.1	41.5	28.4	8.6	<0.01	Adora3	0	NM_027025
chr3	10578020 1	10578110 0	900	HYPO	24.1	18.5	8.9	30.1	28	40.7	11.6	<0.01	Ovgp1	0	NM_007696
chr3	10829160 1	10829230 0	700	HYPO	41.4	42.2	56.8	47.1	50.4	55.2	9.1	<0.01	5330417C22Rik	0	NM_001033304
chr3	13741540 1	13741650 0	1100	HYPO	14.5	16.8	17.9	30.6	28.5	27.2	13.1	<0.01	.	.	NM_016750
chr3	14811720 1	14811810 0	900	HYPO	56.2	52.2	68.2	59.3	62.8	72.7	10.7	<0.01	.	.	NM_001081298
chr4	10832001	10832900	900	HYPO	31.9	25.5	32.4	44.3	41.6	34	11.3	<0.01	2610301B20Rik	5431	NM_026005
chr4	13293601	13294300	700	HYPO	45.5	49.2	38.5	62.5	59.5	61.9	14.0	<0.01	.	.	NM_001111026
chr4	21841201	21842300	1100	HYPO	44.4	46.7	60	60.4	49.6	55.7	9.7	<0.01	Coq3	1928	NM_172687
chr4	59732601	59733700	1100	HYPO	56.3	47.7	52.6	75.8	51	61.3	10.7	<0.01	E130308A19Rik	0	NM_153158
chr4	65042201	65042900	700	HYPO	10	9.6	40	22.5	12.5	36.6	14.1	<0.01	Astn2	0	NM_019514
chr4	88768801	88769700	900	HYPO	55.5	45.7	28.6	66.4	69.5	72.3	18.8	<0.01	Mtap	-	NM_024433

														13573	
	10615740	10615830													
chr4	1	0	900	HYPO	72	76.6	100	91.9	86.1	80.4	11.8	<0.01	Bsnd	0	NM_080458
	10628620	10628730	110												
chr4	1	0	0	HYPO	63.9	52	66	69.2	74.2	73.3	14.3	<0.01	BC055111	0	NM_183182
	10930860	10930950													
chr4	1	0	900	HYPO	50.7	48.4	55.7	62	59.6	59.9	11.0	<0.01	Cdkn2c	23980	NM_007671
	12595620	12595710													
chr4	1	0	900	HYPO	36.7	34.3	38.9	52.4	59.3	49.8	18.3	<0.01	Trappc3	3074	NM_013718
	12853860	12854010	150											-	
chr4	1	0	0	HYPO	36.1	44.5	36.1	45.8	48.3	50.5	7.9	<0.01	Trim62	21283	NM_178110
	12993980	12994150	170												NM_00125338
chr4	1	0	0	HYPO	85	82.9	95	94.3	93.3	94.4	10.1	<0.01	Serinc2	0	6
	14347160	14347250													NM_00108541
chr4	1	0	900	HYPO	65.8	65.5	79.5	73.2	75.1	73.6	8.3	<0.01	Pramef6	11639	4
	15435780	15435870													NM_00111336
chr4	1	0	900	HYPO	20.4	20	.	37.4	30.5	33.9	13.7	<0.01	Plch2	0	0
	15562800	15562890												-	
chr4	1	0	900	HYPO	26.9	26.6	.	42.6	45	40.8	16.1	<0.01	Klhl17	19035	NM_198305
			110												
chr5	16435401	16436500	0	HYPO	46.6	43.8	25	68.1	48	58.5	13.0	<0.01	.	.	NM_010427
			150												
chr5	31678001	31679500	0	HYPO	84.1	78.8	83.3	90.9	91.4	95	11.0	<0.01	Gckr	48326	NM_144909
chr5	38691001	38691900	900	HYPO	8.6	10.3	11.4	14.6	9.8	23.1	6.4	<0.01	Otop1	0	NM_172709
chr5	65736801	65737500	700	HYPO	4.8	5.6	.	15	9.5	17.4	8.8	<0.01	Klb	-2149	NM_031180
															NM_00119073
chr5	73963001	73963900	900	HYPO	76.2	71	82.2	89.5	93.8	89.5	17.3	<0.01	Dcun1d4	10968	4
	10008220	10008310											A930011G23Ri		
chr5	1	0	900	HYPO	26.3	26.6	29.4	48.6	40.8	25.5	11.9	<0.01	k	0	NR_030692
	10541220	10541310													
chr5	1	0	900	HYPO	20.9	19.6	30	33.7	27.7	47.2	16.0	<0.01	Abcg3	-465	NM_030239
	10776020	10776130	110												NM_00107987
chr5	1	0	0	HYPO	13.7	16.2	20.4	24.5	29.8	23.6	11.0	<0.01	Brdt	0	3
	10869120	10869210													
chr5	1	0	900	HYPO	37.3	41.8	41.1	52.7	46.3	57.7	12.7	<0.01	Dr1	-5815	NM_026106
	11371740	11371810													
chr5	1	0	700	HYPO	58.3	61.8	.	75.5	70.9	66.7	11.0	<0.01	Sgsm1	0	NM_172718
	11807940	11808090	150												NM_00111454
chr5	1	0	0	HYPO	16.1	17	5.5	29.7	24.4	25	9.8	<0.01	Ksr2	0	5
	11957520	11957610											1700081H04Ri		
chr5	1	0	900	HYPO	30.2	49.4	52.5	58.4	51.6	63.3	18.0	<0.01	k	10649	NR_040693
	12528340	12528430													
chr5	1	0	900	HYPO	32.8	30.4	41.1	48.9	37.2	41.1	10.8	<0.01	D.hc10	0	NM_019536
chr5	12945300	12945410	110	HYPO	12.8	10.9	22.2	21.3	18	23.6	9.1	<0.01	Rimbp2	0	NM_00108138

	1	0	0												8
chr5	13499840 1	13499910 0	700	HYPO	27.8	25.4	32.6	41.4	33.4	43.6	12.9	<0.01	Clip2	0	NM_00103916 2
chr5	13727860 1	13727930 0	700	HYPO	11.2	4.7	10.7	15	14.3	41.6	15.7	<0.01	Emid2	0	NM_024474
chr5	14858640 1	14858750 0	110 0	HYPO	31.3	16.7	.	61.2	64.2	48.1	33.8	<0.01	Flt1	-48837	NM_010228
chr6	53635001	53635900	900	HYPO	61	65.2	48.2	62.5	84.8	73	10.3	<0.01	Creb5	0	NM_172728
chr6	82866801	82868100	130 0	HYPO	71.7	76	.	82.4	89	90.9	13.6	<0.01	Sema4f	0	NM_011350
chr6	11422200 1	11422290 0	900	HYPO	68.4	68.2	72.3	81.6	77.2	72.5	8.8	<0.01	Slc6a1	-9728	NM_178703
chr6	11712580 1	11712670 0	900	HYPO	58.8	63.7	54.8	73.2	64.6	80.2	11.4	<0.01	Cxcl12	0	NM_00101247 7
chr6	12472020 1	12472110 0	900	HYPO	86.1	83.8	85.8	93.5	96.8	92	9.2	<0.01	Lrrc23	0	NM_013588
chr6	12555280 1	12555410 0	130 0	HYPO	36.9	32.9	27.7	39.8	39.3	51.2	8.5	<0.01	Vwf	0	NM_011708
chr6	12696000 1	12696090 0	900	HYPO	73.6	78.5	94.7	94.5	82.2	84	10.9	<0.01	Fgf6	-4659	NM_010204
chr6	13499020 1	13499090 0	700	HYPO	10.7	10.7	10.5	20.8	11	25.3	8.3	<0.01	Ddx47	16407	NM_026360
chr6	14019080 1	14019170 0	900	HYPO	55.1	41.8	53.9	68.7	62.9	67.8	18.0	<0.01	.	.	NM_144920
chr6	14529540 1	14529710 0	170 0	HYPO	64.2	61.1	69.8	75.6	74.1	77.9	13.2	<0.01	1700073E17Rik	-39044	NR_003625
chr7	4780801	4781900	110 0	HYPO	31.4	38.9	46.2	41.1	51.4	61.5	16.2	<0.01	Shisa7	0	NM_172737
chr7	28160001	28160700	700	HYPO	0	2.9	.	.	12.1	.	10.7	<0.01	Spnb4	0	NM_00119923 5
chr7	46914001	46915100	110 0	HYPO	32.9	30.8	26.5	40.8	47.8	36.8	10.0	<0.01	.	.	NM_00100413 9
chr7	53285201	53286300	110 0	HYPO	69.1	63	40	100	88.9	76.4	22.4	<0.01	Abcc6	0	NM_018795
chr7	55765601	55766500	900	HYPO	62.3	68.2	43.2	68.5	94.1	73	13.3	<0.01	Mrgprx2	-10961	NM_00103486 8
chr7	66313601	66314700	110 0	HYPO	66.4	81.6	90	86.4	90.4	80	11.6	<0.01	.	.	NM_173010
chr7	80476601	80477500	900	HYPO	50	46.5	64.8	70.8	48.4	61.4	12.0	<0.01	A730056A06Rik	0	NR_040324
chr7	10713860 1	10713970 0	110 0	HYPO	22.2	29.3	9.4	40	39.5	18.2	6.8	<0.01	Rnf169	-9633	NM_175388
chr7	10852100 1	10852230 0	130 0	HYPO	64.5	65.8	60	79.2	72.8	82.4	13.0	<0.01	Arap1	0	NM_00104011 1

chr7	10897200 1	10897290 0	900	HYPO	48.8	52.4	50	68.3	51.1	64.1	10.6	<0.01	Inppl1	0	NM_001122739
chr7	11179540 1	11179650 0	110 0	HYPO	4.5	8.3	.	23.4	26.1	20.3	16.9	<0.01	Olfr658	0	NM_147049
chr7	12007180 1	12007270 0	900	HYPO	22.1	24	37.1	40.4	43.7	21	12.0	<0.01	Tead1	21482	NM_001166584
chr7	12616020 1	12616110 0	900	HYPO	3.1	9.2	5.5	18.1	21.5	15.3	12.2	<0.01	Gprc5b	-21476	NM_001195774
chr7	13618600 1	13618690 0	900	HYPO	21.6	20	8	29.2	30	31.3	9.4	<0.01	.	.	NM_001080963
chr7	13828160 1	13828250 0	900	HYPO	33.2	32.9	41.4	41.6	44.8	46.3	11.2	<0.01	4933402N03Rik	0	NM_173409
chr8	12486401	12487500	110 0	HYPO	64.2	60.8	72.4	73.3	69.9	73.7	9.8	<0.01	Gm5607	49669	NR_027975
chr8	16297201	16297900	700	HYPO	28.1	28.1	.	40	42.9	43.8	14.1	<0.01	Csmd1	0	NM_053171
chr8	32849801	32850700	900	HYPO	16.1	14.9	17.2	24	28.7	23.6	9.9	<0.01	Nrg1	77799	NM_178591
chr8	43268801	43269700	900	HYPO	57.9	48.5	41	65.6	61.7	71.2	13.0	<0.01	.	.	NR_045497
chr8	44248201	44249500	130 0	HYPO	13.6	13.8	11.7	15.1	20.6	10.9	1.8	<0.01	Triml2	-18975	NM_001160412
chr8	45486601	45487500	900	HYPO	63.5	60.3	50	76	63.1	82	11.8	<0.01	.	.	NM_001081286
chr8	59362001	59362900	900	HYPO	13.3	14.8	.	20.5	27.6	36.1	14.0	<0.01	.	.	NM_015791
chr8	69080801	69081900	110 0	HYPO	13.8	16.5	15.5	30.1	24.5	30.9	13.4	<0.01	Tktl2	37703	NM_001271574
chr8	89288001	89289300	130 0	HYPO	23.5	18.8	31	42.3	26.9	25.6	10.5	<0.01	Gm10638	17042	NR_027829
chr8	94729001	94729900	900	HYPO	44.9	48.4	73.5	58	73.5	57.6	16.4	<0.01	.	.	NR_033641
chr8	11301000 1	11301070 0	700	HYPO	31.1	30.9	33.3	56.8	41.4	39.3	14.8	<0.01	Hydin	0	NM_172916
chr8	11902820 1	11902910 0	900	HYPO	14.5	16.7	20	26	13.7	38.3	10.4	<0.01	Dynlrb2	0	NM_029297
chr8	12572560 1	12572650 0	900	HYPO	90.9	90.5	.	100	100	.	9.3	<0.01	Dpep1	0	NM_007876
chr9	22403601	22404500	900	HYPO	44.1	43.4	54.2	51.6	51.7	58.7	10.3	<0.01	Bbs9	0	NM_178415
chr9	63973601	63974300	700	HYPO	13.5	13.1	2.4	25.2	18.3	27.3	10.3	<0.01	Lctl	0	NM_145835
chr9	83282601	83283700	110 0	HYPO	62.2	66.8	81.8	82.4	75	75.3	13.1	<0.01	Lca5	1261	NM_029434
chr9	10094540 1	10094690 0	150 0	HYPO	74.2	69.6	76.2	85.2	80.1	83.5	11.0	<0.01	Pccb	-10107	NM_025835
chr9	10244760 1	10244870 0	110 0	HYPO	38.5	32.2	30.3	47.3	38.9	57	12.4	<0.01	Ky	0	NM_024291
chr9	10392320 1	10392410 0	900	HYPO	47.1	39.7	48.7	58.5	57.2	56.7	14.1	<0.01	Nphp3	0	NM_028721

chr9	108750401	108751500	1100	HYPO	39.3	39.6	16.4	52.2	50.1	45.6	9.9	<0.01	Celsr3	0	NM_080437
chr9	114300601	114301300	700	HYPO	33.3	35.3	33.3	52.4	41.1	54.9	15.2	<0.01	Crtap	-771	NM_019922
chr10	13131801	13132900	1100	HYPO	46.9	48.2	49.7	56.9	54.5	58	8.9	<0.01	Phactr2	0	NM_001195065
chr10	27677201	27678100	900	HYPO	16.7	18.5	.	30.4	25	27.8	10.1	<0.01	.	.	NM_008983
chr10	39977201	39977900	700	HYPO	15	10.5	17.9	27.1	24	19.5	10.8	<0.01	Gtf3c6	0	NM_026113
chr10	40530201	40531100	900	HYPO	40.1	35.2	27.3	56.3	37.4	66.5	15.8	<0.01	9030224M15Rik	0	NM_177793
chr10	51383401	51384300	900	HYPO	29.2	32.3	36.4	46.9	39.2	45.5	13.1	<0.01	Rfx6	-132659	NM_001159389
chr10	59707601	59708500	900	HYPO	22.6	16	27.4	34.8	32.2	25.7	11.6	<0.01	Chst3	-25593	NM_016803
chr10	62328601	62329500	900	HYPO	45.6	48.7	54.2	66.4	72.6	61.5	19.7	<0.01	Tet1	0	NM_001253857
chr10	75328401	75329300	900	HYPO	51.1	48.5	46.3	63.1	54.6	62.9	10.4	<0.01	Mif	-5406	NM_010798
chr10	75344601	75345700	1100	HYPO	20.1	20.6	27.9	25.1	23.6	41.5	9.7	<0.01	Derl3	-10442	NM_024440
chr10	78559801	78560700	900	HYPO	2.8	6.3	3	23.4	9.6	9.8	9.7	<0.01	2610008E11Rik	0	NM_001004362
chr10	79488601	79489500	900	HYPO	13.1	10.4	16.6	24	17.4	35.2	13.8	<0.01	Hmha1	0	NM_001142701
chr10	80107001	80107900	900	HYPO	16.2	22.9	9.9	21	31.9	29.5	7.9	<0.01	Btbd2	0	NM_145361
chr10	80889401	80890300	900	HYPO	6.3	10.1	0	17.9	7.4	0	0.2	<0.01	Nfic	0	NM_008688
chr10	80958001	80959100	1100	HYPO	23	17.4	11.3	32	28.5	33.1	11.0	<0.01	Ncln	0	NM_134009
chr10	120596801	120597900	1100	HYPO	74	81.9	95.5	98.1	98.2	97.4	20.0	<0.01	Wif1	59103	NM_011915
chr10	123891201	123892100	900	HYPO	86.9	86.8	100	100	91.9	95.2	8.9	<0.01	4930503E24Rik	-14029	NR_028310
chr11	3035201	3036100	900	HYPO	77.2	76.7	66.7	79	87.5	89.8	8.5	<0.01	Sfi1	0	NM_030207
chr11	4799401	4800300	900	HYPO	22.4	25.9	11.1	45.1	34	37.5	14.7	<0.01	Thoc5	0	NM_172438
chr11	57262201	57263100	900	HYPO	10.9	8.3	4	8	28	42.9	16.7	<0.01	Fam114a2	33391	NM_001168667
chr11	58886201	58887300	1100	HYPO	86.2	83.3	.	97.9	96.2	94.5	11.5	<0.01	Obscn	0	NM_001171512

chr1	60309201	60310100	900	HYPO	37.3	36.4	49.3	45.6	52.9	49.1	12.4	<0.01	Myo15	0	NM_001103171
chr1	70142001	70142900	900	HYPO	32.6	31.9	24.9	38.3	42.8	42.8	9.1	<0.01	Alox12e	-5981	NM_145684
chr1	73891201	73892100	900	HYPO	20.2	12.3	15.4	19.7	27.8	37.7	12.2	<0.01	Zfp616	0	NM_001177570
chr1	75078401	75079500	1100	HYPO	73.4	72.8	86.3	81.9	85.8	88.1	12.2	<0.01	Rtn4rl1	0	NM_177708
chr1	80963801	80964700	900	HYPO	36.1	36.1	45	48.7	45	49.8	11.7	<0.01	Accn1	0	NM_007384
chr1	113571401	113572300	900	HYPO	69.7	70.2	.	85.2	86.1	72	11.2	<0.01	Cpsf4l	-70	NM_029794
chr1	113646801	113647700	900	HYPO	65.4	60	.	71.7	66.2	83.2	11.0	<0.01	Sdk2	0	NM_172800
chr2	60837801	60838700	900	HYPO	38.8	37.3	38.2	40.3	54.1	48	9.4	<0.01	.	.	NM_001033156
chr2	99926601	99928100	1500	HYPO	45.8	45.5	43.3	53	52.6	56.6	8.4	<0.01	Ptpn21	0	NM_011877
chr2	105521201	105521900	700	HYPO	75.7	80	.	76.2	100	100	14.2	<0.01	Serpi.3i	13626	NM_001199940
chr2	111096601	111097500	900	HYPO	46.7	53.8	55.1	78.6	45.9	66.6	13.5	<0.01	.	.	NR_028265
chr3	80712001	80721000	900	HYPO	55.2	55.7	63	63.1	72.5	72.8	14.0	<0.01	.	.	NM_052977
chr3	234868001	234887000	1900	HYPO	16.3	17.1	14.2	33.4	25.4	17.5	8.7	<0.01	Abt1	21529	NM_013924
chr3	314052001	314063000	1100	HYPO	42.7	38.6	19.6	48.6	49.4	60.5	12.2	<0.01	4930401O12Rik	-72246	NR_045957
chr3	314202001	314209000	700	HYPO	65.6	57.6	47.6	72.4	72.8	88	16.1	<0.01	4930401O12Rik	-87246	NR_045957
chr3	392656001	392665000	900	HYPO	31.7	28.1	19.2	44	36.6	34.8	8.6	<0.01	.	.	NM_001170431
chr3	491046001	491055000	900	HYPO	21.7	27.7	25	47.3	37.9	29.5	13.5	<0.01	Wnk2	26170	NM_029361
chr3	580494001	580501000	700	HYPO	63	56.5	.	70.1	82.3	67.7	13.6	<0.01	Spock1	-39708	NM_001166464
chr3	982046001	982055000	900	HYPO	15.9	16.1	22.4	26	23.9	22.8	8.2	<0.01	.	.	NR_046157
chr4	486012001	486023000	1100	HYPO	57.4	60.1	54.8	71	65.1	68.8	9.6	<0.01	4930447J18Rik	37337	NR_045959
chr4	525136001	525143000	700	HYPO	16.4	9.6	12.5	25	29.6	.	14.3	<0.01	Slc39a2	0	NM_001039676
chr4	550946001	550955000	900	HYPO	20	16.1	.	25	25	39	11.6	<0.01	Rem2	0	NM_080726

chr14	86143801	86144900	1100	HYPO	84.7	82.7	87.5	96.2	92	93.1	10.1	<0.01	.	.	NR_040457
chr15	28276201	28277100	900	HYPO	11.1	12.4	28.3	23.4	21.4	16.1	8.6	<0.01	D.hc5	0	NM_133365
chr15	62397401	62398300	900	HYPO	57	41.8	40.8	78.9	69.3	33.3	11.1	<0.01	.	.	NR_003368
chr15	69715201	69716100	900	HYPO	68.4	69.5	81.8	77.4	75.1	81.7	9.1	<0.01	Gm19782	-38562	NR_045071
chr15	79922401	79923700	1300	HYPO	34.9	36.7	50	47.5	43.5	47.6	10.4	<0.01	Syngr1	0	NM_009303
chr15	81632601	81633500	900	HYPO	40.9	39.8	.	61.3	49.1	66.7	18.7	<0.01	Tef	0	NM_153484
chr15	93188801	93189700	900	HYPO	53.8	53.2	55	81.8	70	88.9	26.7	<0.01	Yaf2	-21435	NM_024189
chr15	98424401	98425300	900	HYPO	70.9	71.7	47.1	89.8	80.3	65.4	7.2	<0.01	Adcy6	0	NM_007405
chr15	98434401	98435300	900	HYPO	63.1	64.5	50	71.6	86.1	79	15.1	<0.01	Adcy6	0	NM_007405
chr16	5165201	5166300	1100	HYPO	51.2	45.6	60	56.8	59	65.9	12.2	<0.01	Sec14l5	0	NM_001127725
chr16	21653001	21653900	900	HYPO	10.5	0	.	30	14.3	26.2	18.3	<0.01	2510009E07Rik	0	NM_001001881
chr16	30540201	30541300	1100	HYPO	39.3	33.6	39	46	47.4	50.6	11.6	<0.01	Tmem44	0	NM_172614
chr16	31931601	31932500	900	HYPO	48.4	48.3	52.1	50.5	57.1	60	7.5	<0.01	Pigz	-1436	NM_172822
chr16	52254401	52255300	900	HYPO	26.1	25.2	35	29.8	40	36.8	9.9	<0.01	Alcam	0	NM_009655
chr16	68915801	68916700	900	HYPO	35.2	31.7	73.1	72.3	47.4	50	23.1	<0.01	.	.	NM_173069
chr16	95875801	95876700	900	HYPO	17.8	13	28.6	20	30.4	27.9	10.7	<0.01	Ets2	-47313	NM_011809
chr17	4974401	4975100	700	HYPO	31.1	39.4	50.4	50.6	44.5	50.9	13.4	<0.01	Arid1b	-19973	NM_001085355
chr17	37107801	37108700	900	HYPO	12.3	14.5	28.9	22.3	23.3	30.4	11.9	<0.01	Znrd1as	5233	NM_029602
chr17	48596801	48597900	1100	HYPO	47.6	39	51.6	60.7	61.8	56.7	16.4	<0.01	Unc5cl	0	NM_152823
chr17	57632201	57633100	900	HYPO	42.8	47	37.6	61.3	53.7	54.9	11.7	<0.01	Emr1	9249	NM_010130
chr17	85259201	85259900	700	HYPO	49.6	50	67.1	64.3	61	60	12.0	<0.01	1110020A21Rik	56621	NR_027929
chr17	85321601	85323100	1500	HYPO	82.4	86.8	99.3	98.4	95.2	96.1	12.0	<0.01	1110020A21Rik	0	NR_027929

chr17	88034801	88035500	700	HYPO	26.2	22.8	20	46.2	32.6	36.6	14.0	<0.01	Epcam	0	NM_008532
chr18	35808401	35809300	900	HYPO	39	42	42	64.6	46.5	51.8	13.8	<0.01	2010001M09Rik	0	NM_027222
chr18	84028601	84029500	900	HYPO	23.5	23.8	16.8	36.9	27.1	37.6	10.2	<0.01	.	.	NM_001081300
chr19	4041601	4042700	1100	HYPO	9.4	10.8	11.6	25	24.1	4.5	7.8	<0.01	Gstp2	0	NM_181796
chr19	11905601	11906700	1100	HYPO	51	50.6	57.1	62.5	62.9	54.1	9.0	<0.01	Olfr1417	-2087	NM_146936
chr19	24078801	24079700	900	HYPO	6.2	0	.	.	15	.	11.9	<0.01	Fam189a2	0	NM_001114174
chr19	27557401	27558500	1100	HYPO	11.5	12.3	23.5	15.5	25.8	20.4	8.7	<0.01	C030016D13Rik	50280	NR_027987
chr19	30241401	30242500	1100	HYPO	13.8	17.7	22.2	30.1	28.9	24.4	12.1	<0.01	Gldc	0	NM_138595
chr19	34620001	34620900	900	HYPO	58.9	55.5	62.8	67.8	65.9	70	10.7	<0.01	Ifit2	-4283	NM_008332
chr19	38841201	38842300	1100	HYPO	66.6	71.2	79	84.4	79.4	81.9	13.0	<0.01	Plce1	0	NM_019588
chr19	40350201	40351100	900	HYPO	5	5	.	3	16.7	4.2	3.0	<0.01	Pdlim1	-4095	NM_016861
chr19	40811001	40811900	900	HYPO	33.6	37.5	45	51.3	51.1	40.2	12.0	<0.01	Entpd1	0	NM_009848
chr19	45072201	45072900	700	HYPO	75	79.2	.	.	.	87.5	10.4	<0.01	Sema4g	0	NM_011976
chr19	47869001	47869900	900	HYPO	14.3	15.6	18.5	48.7	52.7	.	35.8	<0.01	Wdr96	0	NM_027559
chrX	4178601	4179500	900	HYPO	29.9	29.5	48.7	49.1	34.9	49	14.6	<0.01	Gm5925	-40482	NR_040410
chrX	34757401	34758300	900	HYPO	11.5	15.2	0	25.4	28	22.4	11.9	<0.01	Rhox1	0	NM_001025084
chrX	51217001	51217900	900	HYPO	6.7	7.8	.	28.2	9.5	12.7	9.6	<0.01	.	.	NM_011725
chrX	82596001	82596700	700	HYPO	17.2	19.2	0	18.7	29.1	40.9	11.4	<0.01	1600014K23Rik	80371	NM_028046
chrX	85460001	85460900	900	HYPO	63.3	63.5	76.8	73.5	71.4	70.8	8.5	<0.01	Il1rapl1	-99039	NM_001160403
chrX	108351801	108352700	900	HYPO	64.9	66.6	72.9	72.6	74.4	80.2	10.0	<0.01	Cylc1	32588	NM_026134
chrX	150698401	150699300	900	HYPO	47.8	50.8	100	82.5	58.3	61.1	18.0	<0.01	4930524N10Rik	74467	NM_001256259

SA model (79 DMRs)

CHR	START	END	SIZE	METHYLATIO	ETOH-	ETOH-	ETOH-	CTRL-	CTRL-2	CTRL-3	ABS_aveDIFF	P.Valu	GENE	DIST	GenBank

				N	1	2	3	1				e			
chr1	29951601	29952500	900	HYPER	74	75.2	76	57.7	57.3	62.1	16.0	<0.01	.	.	NM_001081080
chr1	50869001	50869900	900	HYPER	47.2	50	54.2	38.2	36.4	31.8	15.0	<0.01	.	.	NM_019790
chr1	66616001	66616900	900	HYPER	62.6	62.9	64.9	60.4	42.9	54.6	10.8	<0.01	Unc80	0	NM_175510
chr2	82714401	82715300	900	HYPER	91.7	94.4	93.3	80	.	.	13.1	<0.01	.	.	NM_175513
chr3	9520201	9521100	900	HYPER	84.6	82.8	86	70.9	75.6	71.3	11.9	<0.01	Zfp704	0	NM_133218
chr3	134347401	134348100	700	HYPER	75.2	75.2	83.3	56.5	75.2	62.5	13.2	<0.01	.	.	NM_021382
chr4	130774201	130775100	900	HYPER	38.3	34.3	33.8	9.1	23.6	22	17.2	<0.01	.	.	NM_010769
chr5	105343801	105344700	900	HYPER	90	87.6	90.1	74.9	71.7	77	14.7	<0.01	Abcg3	19375	NM_030239
chr5	120372201	120373100	900	HYPER	46.4	50.7	51.4	32.4	34.9	40.2	13.7	<0.01	Tbx5	36974	NM_011537
chr6	141652801	141653700	900	HYPER	97.9	100	100	85.1	92.6	89.2	10.3	<0.01	Slco1b2	17645	NM_020495
chr7	25445801	25447300	1500	HYPER	72.4	70.2	72	60.4	63.3	61.7	9.7	<0.01	Gm4598	0	NR_030681
chr7	33276401	33277100	700	HYPER	71.4	74.3	77.3	69.4	57.7	58.9	12.3	<0.01	Scgb1b29	48538	NM_001256066
chr7	51868601	51870100	1500	HYPER	38.9	39.8	41.1	30.2	30.9	29.2	9.8	<0.01	Myh14	0	NM_001271538
chr7	147361801	147362700	900	HYPER	76.8	82	81	58.1	44.4	55.9	27.1	<0.01	Olfr523	0	NM_146518
chr8	46513601	46514300	700	HYPER	88.6	89	90	87.5	81.3	67.5	10.4	<0.01	Tlr3	-17708	NM_126166
chr8	67096001	67096900	900	HYPER	85.4	87.7	93.8	74.8	89.8	66.2	12.0	<0.01	Cpe	0	NM_013494
chr9	72704201	72705100	900	HYPER	54.3	50.1	64.4	41.4	39.4	41.8	15.4	<0.01	Prtg	0	NM_175485
chr10	6389401	6390300	900	HYPER	89.2	93.2	98.6	86.2	76.2	83	11.9	<0.01	Plekhg1	0	NM_001033253
chr10	19924801	19925500	700	HYPER	26.3	31.4	24.4	10.8	10.4	15.4	15.2	<0.01	Mtap7	0	NM_001198635
chr10	58121401	58122300	900	HYPER	87.9	80.4	84.2	84.6	62.4	68.9	12.2	<0.01	Edar	0	NM_010100
chr10	59030001	59030700	700	HYPER	83.4	86.7	93.2	80.2	80	61.3	13.9	<0.01	Ccdc109a	0	NM_001033259
chr10	66181801	66182700	900	HYPER	29.7	27.6	30.3	19.2	12.5	21.9	11.3	<0.01	.	.	NM_001204915
chr11	41553201	41553900	700	HYPER	78.2	79.2	86.7	57.1	65	75.3	15.6	<0.01	.	.	NM_177408
chr11	109106001	109106901	900	HYPER	90.7	91.2	95.8	76.8	80.5	77.8	14.2	<0.01	Rgs9	0	NM_011268

chr12	20077401	20078300	900	HYPER	51.2	50.4	60	33.9	47.7	38.7	13.8	<0.01	.	.	NR_015521
chr12	10779660	10779750	900	HYPER	76.8	79.1	91.1	71.4	65.9	64.4	15.1	<0.01	.	.	NR_036593
chr13	56542001	56542700	700	HYPER	89.8	88.2	88.9	73.6	77	79	12.4	<0.01	Slc25a48	0	NM_177809
chr13	81833201	81834100	900	HYPER	48.6	50.4	53.4	44.8	37.8	30.8	13.0	<0.01	Polr3g	0	NM_001081176
chr16	43969001	43969700	700	HYPER	40	33.5	47.3	26.7	22.5	23.7	16.0	<0.01	Zdhhc23	0	NM_001007460
chr17	25727001	25727900	900	HYPER	85.1	90.9	91.4	77.9	82.7	70.5	12.1	<0.01	Lmf1	0	NM_029624
chr18	3975801	3976700	900	HYPER	75	75	100	56.4	69	63.9	20.2	<0.01	.	.	NM_026092
chr18	53757201	53758300	1100	HYPER	89.2	84.1	86	77	74.1	61.8	15.5	<0.01	Prdm6	21690	NM_001033281
chr18	73595201	73596300	1100	HYPER	60.9	62.8	59.3	56.2	36.3	54.6	12.0	<0.01	.	.	NM_001039214
chr19	48306001	48306900	900	HYPER	94.8	84.9	100	70.4	83.4	69.3	18.9	<0.01	Sorcs3	0	NM_025696
chrX	37252801	37253700	900	HYPER	61.4	58.1	53.3	40.3	30.4	51.6	16.8	<0.01	Cypt14	-36020	NM_001191032
chrX	91792401	91793500	1100	HYPER	47.1	45.5	61.9	44.3	31.3	34.8	14.7	<0.01	Maged1	-4988	NM_019791
chrX	115258801	115259700	900	HYPER	70.7	54	60	47.5	29.3	53	18.3	<0.01	.	.	NM_001142750
chrX	143693601	143694500	900	HYPER	34.6	35.9	43.9	.	17.5	.	20.6	<0.01	Htr2c	61781	NM_008312
chr2	105194601	105194701	110	HYPO	40.8	40.2	36.2	59.9	43.7	47.2	11.2	<0.01	0610012H03Rik	0	NM_001159638
chr2	159053401	159054101	700	HYPO	53.4	53.8	54.1	63.9	66.4	63.5	10.8	<0.01	.	.	NM_145742
chr2	168686401	168687101	700	HYPO	44.4	58.3	47.6	86.7	71.7	78.8	29.0	<0.01	Zfp64	63760	NM_009564
chr3	37498201	37499300	1100	HYPO	27.4	23.3	16.2	44.1	42.2	23.7	14.4	<0.01	Spata5	20184	NM_001163511
chr3	105780201	105781101	900	HYPO	24.1	18.5	8.9	30.1	28	40.7	15.8	<0.01	Ovgp1	0	NM_007696
chr3	137415401	137416501	1100	HYPO	14.5	16.8	17.9	30.6	28.5	27.2	12.4	<0.01	.	.	NM_016750
chr4	132936013	132943003	700	HYPO	45.5	49.2	38.5	62.5	59.5	61.9	16.9	<0.01	.	.	NM_001111026
chr4	125956201	125957101	900	HYPO	36.7	34.3	38.9	52.4	59.3	49.8	17.2	<0.01	Trappc3	3074	NM_013718

chr5	108691201	108692100	900	HYPO	37.3	41.8	41.1	52.7	46.3	57.7	12.2	<0.01	Dr1	-5815	NM_026106
chr5	137278601	137279300	700	HYPO	11.2	4.7	10.7	15	14.3	41.6	14.8	<0.01	Emid2	0	NM_024474
chr6	53635001	53635900	900	HYPO	61	65.2	48.2	62.5	84.8	73	15.3	<0.01	Creb5	0	NM_172728
chr6	117125801	117126700	900	HYPO	58.8	63.7	54.8	73.2	64.6	80.2	13.6	<0.01	Cxcl12	0	NM_001012477
chr6	125552801	125554100	1300	HYPO	36.9	32.9	27.7	39.8	39.3	51.2	10.9	<0.01	Vwf	0	NM_011708
chr6	140190801	140191700	900	HYPO	55.1	41.8	53.9	68.7	62.9	67.8	16.2	<0.01	.	.	NM_144920
chr7	46914001	46915100	1100	HYPO	32.9	30.8	26.5	40.8	47.8	36.8	11.7	<0.01	.	.	NM_001004139
chr7	53285201	53286300	1100	HYPO	69.1	63	40	100	88.9	76.4	31.1	<0.01	Abcc6	0	NM_018795
chr7	108972001	108972901	900	HYPO	48.8	52.4	50	68.3	51.1	64.1	10.8	<0.01	Inppl1	0	NM_001122739
chr7	126160201	126161101	900	HYPO	3.1	9.2	5.5	18.1	21.5	15.3	12.4	<0.01	Gprc5b	-21476	NM_001195774
chr8	45486601	45487500	900	HYPO	63.5	60.3	50	76	63.1	82	15.8	<0.01	.	.	NM_001081286
chr8	69080801	69081900	1100	HYPO	13.8	16.5	15.5	30.1	24.5	30.9	13.2	<0.01	Tktl2	37703	NM_001271574
chr9	63973601	63974300	700	HYPO	13.5	13.1	2.4	25.2	18.3	27.3	13.9	<0.01	Lctl	0	NM_145835
chr9	102447601	102448701	1100	HYPO	38.5	32.2	30.3	47.3	38.9	57	14.1	<0.01	Ky	0	NM_024291
chr9	108750401	108751501	1100	HYPO	39.3	39.6	16.4	52.2	50.1	45.6	17.5	<0.01	Celsr3	0	NM_080437
chr9	114300601	114301301	700	HYPO	33.3	35.3	33.3	52.4	41.1	54.9	15.5	<0.01	Crtap	-771	NM_019922
chr10	40530201	40531100	900	HYPO	40.1	35.2	27.3	56.3	37.4	66.5	19.2	<0.01	9030224M15Rik	0	NM_177793
chr10	75328401	75329300	900	HYPO	51.1	48.5	46.3	63.1	54.6	62.9	11.6	<0.01	Mif	-5406	NM_010798
chr10	80107001	80107900	900	HYPO	16.2	22.9	9.9	21	31.9	29.5	11.1	<0.01	Btbd2	0	NM_145361
chr11	3035201	3036100	900	HYPO	77.2	76.7	66.7	79	87.5	89.8	11.9	<0.01	Sfi1	0	NM_030207
chr11	4799401	4800300	900	HYPO	22.4	25.9	11.1	45.1	34	37.5	19.1	<0.01	Thoc5	0	NM_172438
chr11	57262201	57263100	900	HYPO	10.9	8.3	4	8	28	42.9	18.6	<0.01	Fam114a2	33391	NM_001168667
chr11	70142001	70142900	900	HYPO	32.6	31.9	24.9	38.3	42.8	42.8	11.5	<0.01	Alox12e	-5981	NM_145684

chr1														NM_001177570
chr1	73891201	73892100	900	HYPO	20.2	12.3	15.4	19.7	27.8	37.7	12.4	<0.01	Zfp616	0
chr1													4930401O12Rik	-87246	NR_045957
chr1	31420201	31420900	700	HYPO	65.6	57.6	47.6	72.4	72.8	88	20.8	<0.01		
chr1	48601201	48602300	1100	HYPO	57.4	60.1	54.8	71	65.1	68.8	10.9	<0.01	4930447J18Rik	37337	NR_045959
chr1	62397401	62398300	900	HYPO	57	41.8	40.8	78.9	69.3	33.3	14.0	<0.01	.	.	NR_003368
chr1	93188801	93189700	900	HYPO	53.8	53.2	55	81.8	70	88.9	26.2	<0.01	Yaf2	-21435	NM_024189
chr1	98424401	98425300	900	HYPO	70.9	71.7	47.1	89.8	80.3	65.4	15.3	<0.01	Adcy6	0	NM_007405
chr1	57632201	57633100	900	HYPO	42.8	47	37.6	61.3	53.7	54.9	14.2	<0.01	Emr1	9249	NM_010130
chr1	35808401	35809300	900	HYPO	39	42	42	64.6	46.5	51.8	13.3	<0.01	2010001M09Rik	0	NM_027222
chr1	84028601	84029500	900	HYPO	23.5	23.8	16.8	36.9	27.1	37.6	12.5	<0.01	.	.	NM_001081300
chr1	47869001	47869900	900	HYPO	14.3	15.6	18.5	48.7	52.7	.	34.6	<0.01	Wdr96	0	NM_027559

CAM model (266 DMRs)

CHR	START	END	SIZE	METHYLATION	ETOH-4	ETOH-5	CTRL-4	CTRL-5	ABS_aveDIFF	P.Value	GENE	DIST	GenBank
chr1	5160001	5161300	1300	HYPER	77.6	79.2	65.5	61.5	14.9	<0.001	Atp6v1h	7371	NM_133826
chr1	157094001	157094900	900	HYPER	100	100	89.8	95.8	10.45	<0.001	Xpr1	27886	NM_011273
chr1	168728601	168729500	900	HYPER	30.3	29.7	16.5	22.4	13.4	<0.001	.	.	.
chr1	179393001	179393700	700	HYPER	39.1	36	25.5	24.5	12.55	<0.001	Zfp238	12104	NM_001012330
chr2	6010801	6011700	900	HYPER	94.4	91.7	80	.	13.05	<0.001	5430407P10Rik	7845	NM_001159657
chr2	25316001	25317300	1300	HYPER	26.6	19.8	5	4.5	17.1	<0.001	BC029214	0	NM_153557
chr2	32737801	32738900	1100	HYPER	20	17.9	9.3	7	10.75	<0.001	Fam129b	0	NM_146119
chr2	33501801	33502900	1100	HYPER	33.5	39.8	26.9	21.2	12.65	<0.001	C130021I20Rik	618	NR_046275
chr2	61877801	61878700	900	HYPER	88.9	80.2	62.6	62.7	20.45	<0.001	Slc4a10	-5871	NM_001242378
chr2	152796401	152797300	900	HYPER	82.4	90	61.6	68.3	21.2	<0.001	Ttll9	0	NM_029064

chr2	16754720 1	16754810 0	900	HYPER	84.3	87.7	72.4	76	11.8	<0.001	A530013C23Rik	24288	NR_015500
chr2	17294360 1	17294450 0	900	HYPER	20.4	18.6	9.1	9.8	10.1	<0.001	Ctcfl	0	NM_001081387
chr2	17308460 1	17308530 0	700	HYPER	76.7	73.3	61.1	66.7	11.1	<0.001	Pmepa1	0	NM_022995
chr3	28590001	28590900	900	HYPER	56.9	47.9	37.3	38.4	16.75	<0.001	Slc2a2	-5924	NM_031197
chr3	12167020 1	12167110 0	900	HYPER	16.2	22.9	5	8.3	11.75	<0.001	Arhgap29	0	NM_172525
chr3	15291860 1	15291930 0	700	HYPER	92.5	93.6	79.8	81.3	12.55	<0.001	St6galnac3	0	NM_011372
chr4	77839201	77840100	900	HYPER	66.5	58.3	47.4	48.3	13.45	<0.001	.	.	.
chr4	10584940 1	10585050 0	110 0	HYPER	91.7	90.9	73.8	81.9	13.5	<0.001	.	.	.
chr4	12552760 1	12552890 0	130 0	HYPER	42.9	40.2	29.8	29.9	11.75	<0.001	2610028E06Rik	36194	NR_015560
chr5	23771801	23772900	110 0	HYPER	43	40.4	26.4	19.1	18.9	<0.001	Mir684-1	8371	NR_030454
chr5	10281700 1	10281790 0	900	HYPER	97.2	95.6	82.6	80.3	14.9	<0.001	Arhgap24	-92509	NM_029270
chr5	10998220 1	10998350 0	130 0	HYPER	26.9	33.6	13.4	15.8	15.95	<0.001	Crlf2	227	NM_001164735
chr5	11156860 1	11156950 0	900	HYPER	100	99.6	89.7	98.5	10.2	<0.001	Ttc28	0	NM_001267622
chr5	11162480 1	11162570 0	900	HYPER	32.4	43.3	22.8	22.4	15.25	<0.001	Ttc28	0	NM_001267622
chr5	11885940 1	11886030 0	900	HYPER	90	85.7	72.7	66.6	18.15	<0.001	.	.	.
chr5	11929740 1	11929810 0	700	HYPER	100	100	.	89.3	12.5	<0.001	Med13l	81955	NM_172424
chr5	12632980 1	12633070 0	900	HYPER	31.6	31.3	18.6	3.3	17.35	<0.001	Tmem132b	56848	NM_001190352
chr5	14735360 1	14735430 0	700	HYPER	81.7	86.5	75	65	14.1	<0.001	Gm3409	1598	NM_001243113
chr6	19538601	19539500	900	HYPER	92.2	90.3	82	80.9	12.7	<0.001	.	.	.
chr6	11154340 1	11154430 0	900	HYPER	70.4	75.4	60.1	53.6	19.4	<0.001	Grm7	26177	NM_177328
chr6	11808860 1	11808950 0	900	HYPER	12.5	12	2.5	2	11.2	<0.001	Csgalnact2	0	NM_030165
chr6	12206420 1	12206510 0	900	HYPER	97.2	95.5	.	83.3	13.05	<0.001	Gm10319	-21545	NR_003624
chr7	17468401	17469300	900	HYPER	97	100	.	86.7	11.8	<0.001	Dact3	0	NM_001081655
chr7	38659201	38660100	900	HYPER	73.5	75.9	66.3	54.2	14.5	<0.001	C80913	84910	NM_011274

chr7	84532601	84533700	1100	HYPER	98.3	96.5	88	91	10.5	<0.001	.	.	.
chr7	152507401	152508500	1100	HYPER	56.2	52.1	41.4	44.7	10.95	<0.001	Mrgprd	0	NM_203490
chr8	12739401	12740300	900	HYPER	98	98.4	85	98.2	12.9	<0.001	Atp11a	-16715	NM_015804
chr8	48896001	48896900	900	HYPER	61.9	58.3	44	47.8	11.6	<0.001	Cldn24	-10596	NM_001111318
chr8	66355601	66356500	900	HYPER	48.8	33.3	22.6	16.7	21.35	<0.001	Gm4975	46990	NM_001195687
chr8	92095601	92096700	1100	HYPER	25.5	25.8	15.1	4.8	11.65	<0.001	.	.	.
chr8	111873001	111873900	900	HYPER	100	100	88.9	.	11.1	<0.001	Gm1943	-8193	NR_002928
chr9	8544001	8544900	900	HYPER	18.6	19.7	0	4.2	14.95	<0.001	Trpc6	0	NM_013838
chr9	13977801	13978700	900	HYPER	41.6	41	17.8	27.2	22.2	<0.001	.	.	.
chr9	89305801	89306900	1100	HYPER	98.9	100	89.2	98.3	11.35	<0.001	.	.	.
chr9	104750001	104750901	900	HYPER	100	98.6	91.3	84.4	11.4	<0.001	Cpne4	0	NM_028719
chr10	34017001	34017900	900	HYPER	20	16.7	9	3.3	12.15	<0.001	Tspyl4	0	NM_030203
chr10	37573001	37573900	900	HYPER	96.9	100	85	.	13.45	<0.001	.	.	.
chr10	81034801	81035900	1100	HYPER	49.1	50.9	36.1	36.4	13.7	<0.001	Tle2	-2131	NM_001252401
chr10	121129801	121130701	900	HYPER	15.2	15.4	0	0	15.3	<0.001	D930020B18Rik	0	NM_177335
chr11	23157001	23157700	700	HYPER	22.2	24.1	11.1	5.6	14.85	<0.001	Xpo1	0	NM_001035226
chr11	44821201	44822100	900	HYPER	46.4	48	40.5	29.4	12.2	<0.001	Ebf1	2527	NM_007897
chr11	63924201	63925300	1100	HYPER	20	16.4	4	8.5	12.7	<0.001	2810001G20Rik	27440	NR_033780
chr11	68691001	68691900	900	HYPER	92.6	91.8	82.3	.	11.65	<0.001	Rnf222	-10154	NM_177060
chr11	79121201	79121900	700	HYPER	94.4	94.5	75.6	87.5	19.15	<0.001	Nf1	-31493	NM_010897
chr12	19723001	19723900	900	HYPER	92.9	95.3	80.8	85.4	11	<0.001	.	.	.
chr12	85702401	85703300	900	HYPER	30.3	26.7	16.4	17.1	11.8	<0.001	Fam161b	0	NM_172581
chr12	113748401	113749501	1100	HYPER	24.5	31.2	11.2	20.2	15.55	<0.001	Tmem179	0	NM_178915
chr12	12570201	12571700	150	HYPER	78.9	85.1	69.3	70.4	11.95	<0.001	Edaradd	0	NM_133643

3			0										
chr13	109100601	109101700	1100	HYPER	54.8	58.2	42.4	43.8	13.4	<0.001	Depdc1b	-4830	NM_178683
chr13	113308001	113308900	900	HYPER	69.7	64.5	54.1	50.4	13.6	<0.001	Il31ra	4115	NM_139299
chr14	31371801	31372700	900	HYPER	73.5	79.8	68.3	59.6	12.75	<0.001	Tkt	0	NM_009388
chr14	62199001	62199700	700	HYPER	91.5	88.9	79	74.2	13.6	<0.001	Trim13	-17362	NM_001164220
chr14	80262001	80262900	900	HYPER	93	92.7	87.5	20	18.85	<0.001	Gm10845	0	NR_033535
chr14	91574201	91575100	900	HYPER	90	86.7	56.3	.	32.05	<0.001	.	.	.
chr15	44924001	44924900	900	HYPER	26.7	33.3	9.1	.	20.9	<0.001	Kcnv1	12929	NM_026200
chr15	65781001	65781900	900	HYPER	99.2	98.1	88.1	.	10.55	<0.001	Hhla1	0	NM_001145096
chr15	66870601	66872300	1700	HYPER	30.4	29.4	18.8	17.6	11.7	<0.001	St3gal1	62136	NM_009177
chr15	100443401	100444300	900	HYPER	21.2	24.1	9	13.7	11.35	<0.001	C330013E15Rik	270	NR_045701
chr16	18976201	18977500	1300	HYPER	19.6	21	7.8	12.5	12.3	<0.001	Hira	5800	NM_010435
chr16	68915801	68916700	900	HYPER	65.6	62.2	54.3	47.2	13.2	<0.001	.	.	.
chr16	91069001	91069900	900	HYPER	53.6	57.3	42.3	32.9	17.85	<0.001	4932438H23Rik	0	NM_001163695
chr16	96906401	96907300	900	HYPER	81.4	76.1	61.1	64.1	16.15	<0.001	Dscam	0	NM_031174
chr17	29334201	29335100	900	HYPER	100	100	88.9	.	11.1	<0.001	Cpne5	0	NM_153166
chr17	32845201	32845900	700	HYPER	100	95.2	61.1	.	36.5	<0.001	Cyp4f15	4907	NM_134127
chr17	45513001	45513900	900	HYPER	41.4	38.7	21.8	48.6	16.25	<0.001	Cdc5l	14938	NM_152810
chr17	46584201	46585100	900	HYPER	25.2	25.2	11.1	16.7	11.3	<0.001	Ttbk1	0	NM_001162864
chr18	3051401	3052300	900	HYPER	91.6	88.7	75.6	80.7	12.05	<0.001	Vmn1r238	70191	NM_001167539
chr18	37912201	37913300	1100	HYPER	27.9	26.1	11.2	18.6	12.1	<0.001	Pcdhgb4	0	NM_033576
chr18	59117401	59118300	900	HYPER	56.6	62.8	46.9	43.9	14.4	<0.001	Adamts19	0	NM_175506
chr19	67816001	67817100	110	HYPER	75.7	73.4	57.2	67.2	12.35	<0.001	Psmg2	2185	NM_134138

8			0										
chr18	84166001	84166900	900	HYPER	35.2	38.5	18.3	29.2	13.05	<0.001	Tshz1	14118	NM_001081300
chr19	8492401	8493100	700	HYPER	61.8	60.7	49.9	48.8	11.85	<0.001	Slc22a30	-12806	NM_177002
chr19	44746401	44747100	700	HYPER	15.3	11.1	3.3	0	11.5	<0.001	Pax2	-84783	NM_011037
chr19	45072201	45072900	700	HYPER	80	82.2	66.7	66.7	14.4	<0.001	Sema4g	0	NM_011976
chrX	5300001	5300900	900	HYPER	76.2	75.4	60.6	57.3	16.9	<0.001	Gm14374	51857	NM_001085523
chrX	126533601	126534301	700	HYPER	83.3	90	.	60	26.65	<0.001	Diap2	0	NM_172493
chrX	159916601	159917301	700	HYPER	20.5	21.3	8.4	13.3	10	<0.001	Grpr	34535	NM_008177
chr1	36338601	36339500	900	HYPO	32.4	25.5	45.1	45.6	16.45	<0.001	Neurl3	-8331	NM_153408
chr1	55277001	55277900	900	HYPO	59.9	61.1	71.4	72.9	11.7	<0.001	Rftn2	0	NM_028713
chr1	75500001	75500900	900	HYPO	13.9	14.6	22.5	14.4	9.55	<0.001	Obsl1	0	NM_178884
chr1	77928601	77929700	1100	HYPO	74	73.1	89.4	89.7	13.75	<0.001	.	.	.
chr1	92056601	92057500	900	HYPO	32.8	33.7	50.7	49.1	16.65	<0.001	Iqca	-6625	NM_029122
chr1	129381201	129382101	900	HYPO	29.2	30.4	41	38.6	10	<0.001	Mgat5	1652	NM_145128
chr1	131839601	131840501	900	HYPO	34.5	35.8	43	47.6	10.05	<0.001	Thsd7b	0	NM_172485
chr1	158989601	158990501	900	HYPO	77.8	79.5	88.9	88.9	10.25	<0.001	1700057K13Rik	38778	NM_028540
chr1	173186401	173187301	900	HYPO	17.2	23.6	32	28.9	9.9	<0.001	Adamts4	0	NM_172845
chr1	188522201	188523101	900	HYPO	31.8	39.3	64.1	66.2	29.45	<0.001	Tgfb2	0	NM_009367
chr2	154030001	154030900	900	HYPO	20.6	19.2	37.3	29.4	13.5	<0.001	.	.	.
chr2	311648001	311655001	7000	HYPO	30.3	24.8	36	47.8	14.35	<0.001	Ncs1	13810	NM_019681
chr2	658570001	658577001	7000	HYPO	42.4	44.2	58.7	55.5	13.85	<0.001	Csrnp3	0	NM_153409
chr2	897428001	897435001	7000	HYPO	77.8	74.1	95.9	93.3	18.65	<0.001	Olfr1257	20887	NM_146982
chr2	105973001	105973901	900	HYPO	35.8	26.5	45.8	45.8	14.65	<0.001	.	.	.
chr2	113837001	113838101	1100	HYPO	56.4	54.8	65.6	68.2	11.3	<0.001	Gjd2	0	NM_010290
chr2	152655001	152655901	900	HYPO	65.6	65	75.8	76.5	10.9	<0.001	Bcl2l1	0	NM_009743
chr2	180965601	180966501	900	HYPO	38	46.3	57.1	63.3	18.05	<0.001	BC006779	0	NM_183162

chr3	8750001	8750900	900	HYPO	59.3	56.8	77.8	86.1	23.85	<0.001	Mrps28	51245	NM_025434
chr3	9520201	9521100	900	HYPO	61.5	60.6	85.8	91.1	28.55	<0.001	Zfp704	0	NM_133218
chr3	38134201	38135100	900	HYPO	23.7	21.4	37.3	29.6	13.15	<0.001	5430434I15Rik	-27160	NR_040541
chr3	88286001	88286900	900	HYPO	27.3	35.1	55.5	41	17.65	<0.001	Lmna	0	NM_001002011
chr3	88452401	88453100	700	HYPO	84	85.2	90.7	97.8	9.7	<0.001	Arhgef2	427	NM_001198911
chr3	12710080 1	12710170 0	900	HYPO	35.1	33.1	48	48.1	14	<0.001	.	.	.
chr3	12988160 1	12988290 0	130 0	HYPO	45.3	41.6	52.9	65.3	14.6	<0.001	Col25a1	-862	NM_001244952
chr3	14451040 1	14451130 0	900	HYPO	15.2	20.3	28.3	31.6	12.15	<0.001	Clca4	0	NM_139148
chr4	9429601	9430500	900	HYPO	29.2	25.1	40.6	26.2	11.95	<0.001	Asph	0	NM_001177849
chr4	41644201	41645100	900	HYPO	32.7	31.8	44.8	46	13.15	<0.001	Cntfr	-78	NM_016673
chr4	50643401	50644300	900	HYPO	53.2	51.9	62.4	63.2	10.25	<0.001	.	.	.
chr4	55762601	55763500	900	HYPO	27.8	32.6	37	46.4	11.5	<0.001	.	.	.
chr4	57351601	57352500	900	HYPO	33.1	32.8	44.8	46.5	12.75	<0.001	1700042G15Rik	20163	NR_038178
chr4	62335001	62335900	900	HYPO	43.4	39.2	56.9	61.8	18.1	<0.001	Rgs3	0	NM_134257
chr4	81455001	81455900	900	HYPO	54.9	49.9	63.5	67.4	13	<0.001	.	.	.
chr4	12558880 1	12559010 0	130 0	HYPO	26.9	25.9	39	36.1	11.1	<0.001	2610028E06Rik	0	NR_015560
chr4	13387180 1	13387270 0	900	HYPO	49.6	51.2	63.4	60	11.3	<0.001	Trim63	0	NM_001039048
chr4	13442360 1	13442450 0	900	HYPO	28.9	27.9	40.3	38.3	10.9	<0.001	Rhd	0	NM_011270
chr4	13547220 1	13547350 0	130 0	HYPO	57.2	55.2	74.7	77.6	20	<0.001	Cnr2	0	NM_009924
chr4	13599900 1	13599990 0	900	HYPO	53.3	53.7	68.7	65.4	13.6	<0.001	Htr1d	0	NM_008309
chr4	13633940 1	13634030 0	900	HYPO	72.1	70.8	82.1	87.5	13.35	<0.001	Ephb2	0	NM_010142
chr4	15003620 1	15003710 0	900	HYPO	69.2	65.6	82.2	78.5	13	<0.001	Slc45a1	-9918	NM_173774
chr4	15135700 1	15135830 0	130 0	HYPO	71	71.4	80.9	81.1	9.8	<0.001	Thap3	0	NM_001145929
chr5	23919401	23920300	900	HYPO	71.6	66.6	80.6	87	14.7	<0.001	Accn3	0	NM_183000
chr5	24244801	24245700	900	HYPO	29.7	34.1	45.7	43.2	12.6	<0.001	Wdr86	-8303	NM_001081441
chr5	44260001	44260900	900	HYPO	16.6	16.2	22.5	31	10.4	<0.001	Cd38	0	NM_007646
chr5	77283601	77284500	900	HYPO	10	8.3	19.1	17.8	12.2	<0.001	C530008M17Ri	0	NM_001163790

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chr5	93415801	93416700	900	HYPO	17.2	16	28.6	33.3	10.4	<0.001	Shroom3	21016	NM_001077596
chr5	97884201	97885900	170 0	HYPO	16.5	17.6	28.6	25.4	9.95	<0.001	Gk2	0	NM_010294
chr5	10064380 1	10064470 0	900	HYPO	62	58.8	79.8	70.5	14.7	<0.001	2310034O05Rik	0	NR_040679
chr5	10356360 1	10356450 0	900	HYPO	62	57.4	71.9	72.4	12.4	<0.001	Mapk10	0	NM_001081567
chr5	10751680 1	10751770 0	900	HYPO	75.9	74.7	83.3	91.4	12	<0.001	Tgfbr3	17888	NM_011578
chr5	11263920 1	11264010 0	900	HYPO	48.5	46.3	57.7	62.8	12.9	<0.001	1700028D13Rik	179	NR_045377
chr5	11360340 1	11360430 0	900	HYPO	50.6	46.7	58.3	61.1	13.85	<0.001	2900026A02Rik	-11068	NM_172884
chr5	11501220 1	11501290 0	700	HYPO	47.8	44	78.3	83.3	31.6	<0.001	BC057022	-5359	NM_001004180
chr5	11538660 1	11538750 0	900	HYPO	50	54.2	66	60.9	11.3	<0.001	Oasl1	0	NM_145209
chr5	11620200 1	11620290 0	900	HYPO	42.1	31.7	55.3	42.5	17.9	<0.001	Ccdc64	-20433	NM_001080808
chr5	11839860 1	11839930 0	700	HYPO	26.7	35.9	48.4	39.2	12.4	<0.001	Nos1	0	NM_008712
chr5	12361000 1	12361110 0	110 0	HYPO	47.3	49	57.3	60.2	10.55	<0.001	Setd1b	0	NM_001040398
chr5	12377400 1	12377510 0	110 0	HYPO	60.2	63.8	74.1	77.2	13.7	<0.001	Bcl7a	-19356	NM_029850
chr5	12396060 1	12396170 0	110 0	HYPO	42.5	45.6	60.2	55.1	13.55	<0.001	B3gnt4	0	NM_198611
chr5	12528340 1	12528430 0	900	HYPO	24.3	28.5	42	42.3	15.8	<0.001	Dnahc10	0	NM_019536
chr5	12976960 1	12977090 0	130 0	HYPO	19.8	18	33.7	29.1	14.8	<0.001	Gpr133	59127	NM_001081342
chr5	13582880 1	13582970 0	900	HYPO	9.1	10.8	23.6	19	11.35	<0.001	Trim50	0	NM_178240
chr5	13801560 1	13801650 0	900	HYPO	11.3	5.5	23.2	9.5	12.7	<0.001	Tfr2	0	NM_015799
chr6	28872201	28873700	150 0	HYPO	50.7	56.7	73.5	73.1	19.6	<0.001	Snd1	33369	NM_019776
chr6	53917401	53918300	900	HYPO	60.9	54.3	79.5	89.1	26.7	<0.001	Cpvl	0	NM_027749
chr6	86183601	86184500	900	HYPO	44.2	54.3	68.8	53.4	16.85	<0.001	Tgfa	0	NM_031199
chr6	12152520 1	12152650 0	130 0	HYPO	63.1	61.3	79.7	78.4	16.9	<0.001	A2m	-59690	NM_175628
chr6	12555280	12555410	130	HYPO	42.8	44.1	60.4	63.3	18.35	<0.001	Vwf	0	NM_011708

	1	0	0										
chr6	14105520 1	14105610 0	900	HYPO	37.6	38.5	59.9	47.5	15.65	<0.001	.	.	.
chr7	19333801	19334700	900	HYPO	29.1	28.1	41.8	44.7	14.7	<0.001	Psg22	21204	NM_001004152
chr7	28247201	28248100	900	HYPO	54.6	55.5	69.8	63	11.45	<0.001	Blvrb	0	NM_144923
chr7	37864001	37864900	900	HYPO	68.5	69.5	76.9	80.2	9.6	<0.001	.	.	.
chr7	75273801	75274700	900	HYPO	13.9	13	26.7	25	12.35	<0.001	Igf1r	0	NM_010513
chr7	76362601	76363500	900	HYPO	84.4	85.4	97.9	96	12.1	<0.001	.	.	.
chr7	83012801	83013500	700	HYPO	71.9	68.1	84.9	81.9	13.3	<0.001	Klhl25	0	NM_001122780
chr7	87731601	87732900	1300	HYPO	69.5	76.8	88.4	81.2	12.2	<0.001	Crtc3	0	NM_173863
chr7	11582180 1	11582270 0	900	HYPO	35.5	30.5	42.3	51.6	13.9	<0.001	Olfr510	9925	NM_146311
chr7	13373160 1	13373250 0	900	HYPO	35.9	33.2	53.4	53.8	19.15	<0.001	Apobr	0	NM_138310
chr7	14812740 1	14812810 0	700	HYPO	20	19.4	30.4	.	10.7	<0.001	Athl1	0	NM_145387
chr7	14985520 1	14985610 0	900	HYPO	39	39.9	50.3	55.2	13.25	<0.001	Igf2as	0	NR_002855
chr8	77907201	77908100	900	HYPO	0	3.1	13.9	.	14.65	<0.001	1700007B14Rik	-64492	NM_001164235
chr8	87334601	87335500	900	HYPO	5.4	5.6	16.7	13.5	9.6	<0.001	Dand5	3803	NM_201227
chr8	90905601	90906500	900	HYPO	16	20	36.5	28.9	14.7	<0.001	Brd7	-19511	NM_012047
chr8	92768401	92769100	700	HYPO	66	65.2	73.9	78.7	10.7	<0.001	Tox3	1910	NM_172913
chr8	11206600 1	11206690 0	900	HYPO	23	26.6	34.7	42	13.5	<0.001	Pmfbp1	0	NM_019938
chr8	11799880 1	11799970 0	900	HYPO	71.5	66.1	84.9	84.6	16	<0.001	.	.	.
chr8	12484720 1	12484810 0	900	HYPO	41.7	56.2	66	73.7	18.1	<0.001	Zfpm1	0	NM_009569
chr8	12524400 1	12524490 0	900	HYPO	42.6	46.6	56.7	62.6	15.1	<0.001	Cbfa2t3	-20992	NM_009824
chr8	12809860 1	12810070 0	210 0	HYPO	34.5	36.6	44.9	48.5	11.15	<0.001	4930567H12Rik	-7837	NR_015535
chr9	6467801	6468700	900	HYPO	82.6	79	96.3	88.6	11.6	<0.001	Pdgfd	90282	NM_027924
chr9	18731401	18732300	900	HYPO	33.3	37	.	46.7	11.55	<0.001	Olfr832	-16793	NM_001011824
chr9	19443401	19444100	700	HYPO	84.4	85.2	96.3	97.4	12.1	<0.001	Zfp317	0	NM_172918
chr9	40198401	40199700	1300	HYPO	11.6	13.5	18.1	21.9	10.65	<0.001	Gramd1b	0	NM_172768
chr9	71464801	71465700	900	HYPO	55.1	54.6	65.7	68.7	12.35	<0.001	Cgnl1	8615	NM_026599

chr9	10798080 1	10798210 0	130 0	HYPO	8.6	9.2	14.3	25.3	17.1	<0.001	Rnf123	0	NM_032543
chr9	11511440 1	11511570 0	130 0	HYPO	48.8	51.8	64.1	57.9	9.8	<0.001	Osbpl10	0	NM_148958
chr9	11956980 1	11957070 0	900	HYPO	56.4	63	71.8	74.8	13.6	<0.001	Scn10a	0	NM_001205321
chr9	12170060 1	12170190 0	130 0	HYPO	63.8	62.5	78.6	71.6	12.1	<0.001	Hhatl	0	NM_029095
chr10	25697801	25698700	900	HYPO	54.2	50	67.5	65.6	14.5	<0.001	Tmem200a	12291	NM_029881
chr10	62328601	62329500	900	HYPO	46.3	47.5	58.9	61.2	13.4	<0.001	Tet1	0	NM_001253857
chr10	66349601	66350900	130 0	HYPO	76.8	83.4	94.4	94.4	14.3	<0.001	.	.	.
chr10	76428801	76429500	700	HYPO	39.5	37.4	53.5	55.1	15.85	<0.001	Pcbp3	-4109	NM_021568
chr10	11711980 1	11712090 0	110 0	HYPO	31.5	18.4	42.4	39.1	24.15	<0.001	Cpm	0	NM_027468
chr10	12678900 1	12678990 0	900	HYPO	52.8	54.2	67.7	60.9	10.8	<0.001	Inhbe	-173	NM_008382
chr11	28969201	28970100	900	HYPO	57.5	58.9	67.2	75.8	13.3	<0.001	Pnpt1	-60650	NM_027869
chr11	48658601	48659500	900	HYPO	41.2	44.7	53.8	54.9	11.45	<0.001	Trim7	0	NM_053166
chr11	49439001	49439900	900	HYPO	70.8	66.6	.	100	31.3	<0.001	Flt4	0	NM_008029
chr11	51712001	51712700	700	HYPO	41.2	47.2	68.6	68	24.1	<0.001	Phf15	-41018	NM_199299
chr11	64333801	64334700	900	HYPO	3.3	0	.	16.7	15.05	<0.001	Hs3st3a1	0	NM_178870
chr11	77787201	77788300	110 0	HYPO	58.4	57.8	71.7	69.9	14	<0.001	Sez6	0	NM_021286
chr11	88687001	88688700	170 0	HYPO	67.7	74	88.8	86.3	16.75	<0.001	Akap1	3405	NM_009648
chr11	99283001	99283900	900	HYPO	41.4	45	62.9	55.1	16.9	<0.001	Krt12	0	NM_010661
chr11	10169120 1	10169250 0	130 0	HYPO	37.3	36.8	41.2	44.3	9.95	<0.001	Etv4	-44577	NM_008815
chr11	10285280 1	10285350 0	700	HYPO	35.4	44	57.9	49.2	13.9	<0.001	Dcakd	1869	NM_026551
chr11	10907980 1	10908070 0	900	HYPO	33.2	53.9	65.1	63.4	18.05	<0.001	Rgs9	5968	NM_011268
chr11	11306960 1	11307110 0	150 0	HYPO	29.1	35.6	41.7	46.7	14.65	<0.001	4732490B19Rik	0	NR_040276

chr1	11692160	11692250									2810008D09Ri		
1	1	0	900	HYPO	64	67.3	80.1	73.8	11.35	<0.001	k	-15596	NR_027059
chr1	11868620	11868730	110										NM_00102493
1	1	0	0	HYPO	56.6	63.8	84.4	88.4	26.2	<0.001	Rbfox3	0	1
chr1													
2	17513601	17514500	900	HYPO	68.5	60.9	75.4	82.6	14.3	<0.001	Odc1	-37178	NM_013614
chr1			130										
2	27745201	27746500	0	HYPO	23.8	28.5	41.8	38.7	14.15	<0.001	Gm20187	28601	NR_045052
chr1			110								1700020O03Ri		
2	87561201	87562300	0	HYPO	3.1	6.1	15.7	6.6	11.1	<0.001	k	-20527	NM_027405
chr1	11379000	11379130	130										
2	1	0	0	HYPO	7.5	9.9	23.9	43.2	16.5	<0.001	Inf2	-35694	NM_198411
chr1													
3	40983001	40983900	900	HYPO	57.7	53.3	50	72.4	15.7	<0.001	Gcnt2	0	NM_023887
chr1													
3	48266801	48267700	900	HYPO	23.7	27.3	33.6	40.2	11.4	<0.001	Id4	-89095	NM_031166
chr1													
3	55267201	55267900	700	HYPO	49.7	48	59.1	68.4	14.85	<0.001	Fgfr4	0	NM_008011
chr1	10028360	10028450											
3	1	0	900	HYPO	30.7	34.8	45.4	36.2	12.65	<0.001	Mtap1b	0	NM_008634
chr1	10138160	10138250											
3	1	0	900	HYPO	17.6	17.2	27.2	27.2	9.8	<0.001	Marveld2	0	NM_178410
chr1													NM_00125335
4	24127801	24128700	900	HYPO	43.2	38.5	58.6	48.8	12.85	<0.001	Kcnma1	0	8
chr1			110										NM_00114602
4	33962401	33963500	0	HYPO	51.6	52.1	55	61	11.95	<0.001	Wdfy4	0	2
chr1													
4	49731801	49732700	900	HYPO	46.4	42.7	56.9	55.3	11.55	<0.001	Mudeng	24403	NM_144535
chr1													
4	60880601	60881500	900	HYPO	35.3	36.4	45.8	45.1	9.65	<0.001	Mtmr6	-2541	NM_144843
chr1	10699240	10699330											
4	1	0	900	HYPO	29.3	29.8	47.3	44.9	18.25	<0.001	.	.	.
chr1			110										
5	9678001	9679100	0	HYPO	7.1	16.4	30.2	25.7	16.15	<0.001	Spef2	0	NM_177123
chr1													
5	34382801	34383500	700	HYPO	10.5	10.6	22.5	32.3	12.35	<0.001	BC030476	0	NM_173421
chr1			110										
5	45629201	45630300	0	HYPO	75.2	73.3	84.7	87.7	11.95	<0.001	.	.	.
chr1													
5	57654801	57655700	900	HYPO	70	74.1	85.8	84.7	13.15	<0.001	Zhx2	0	NM_199449
chr1													
5	75980401	75981100	700	HYPO	16.7	14.3	.	50	34.5	<0.001	Plec	20303	NM_201394
chr1			110										
5	78501801	78502900	0	HYPO	58.9	61.8	75.4	70.7	12.75	<0.001	Elfn2	0	NM_183141

chr15	81094601	81095500	900	HYPO	41.7	41.1	54.9	53.3	12.7	<0.001	Mchr1	25207	NM_145132
chr15	82561601	82562500	900	HYPO	53.9	52.6	64	72.2	14.85	<0.001	Cyp2d40	27762	NM_023623
chr15	89595801	89596500	700	HYPO	0	0	11.1	.	11.1	<0.001	Syt10	16323	NM_018803
chr15	97612601	97613500	900	HYPO	40	43.5	31.1	55.9	13.15	<0.001	Slc48a1	-1295	NM_026353
chr15	99357801	99358700	900	HYPO	5.1	3.7	25.9	0	21.5	<0.001	Faim2	0	NM_001038658
chr16	4578401	4579300	900	HYPO	22.4	32.3	44.9	41.7	15.95	<0.001	Glis2	-15412	NM_031184
chr16	7224201	7225100	900	HYPO	71	79.2	82.5	89.5	11.6	<0.001	Rbfox1	0	NM_021477
chr16	29720001	29720900	900	HYPO	23.8	23.7	43.9	16.6	15.4	<0.001	Opa1	65313	NM_001199177
chr16	30220201	30220900	700	HYPO	66.7	68	77.2	100	10.65	<0.001	Cpn2	35564	NM_027904
chr16	33834001	33834900	900	HYPO	13.1	14.8	.	25	11.05	<0.001	Itgb5	0	NM_001145884
chr16	46257201	46258100	900	HYPO	76.6	81.5	83	94.1	10.2	<0.001	.	.	.
chr16	54642601	54643500	900	HYPO	25	31.1	41.6	43.3	14.45	<0.001	.	.	.
chr16	91591401	91592300	900	HYPO	38.5	42.8	65	67.2	25.45	<0.001	Tmem50b	0	NM_030018
chr16	93438001	93438700	700	HYPO	47.7	43.3	59.5	57.5	13	<0.001	1700029J03Rik	0	NR_040494
chr17	4893801	4894700	900	HYPO	39.1	38	47.4	52.9	11.65	<0.001	.	.	.
chr17	19301201	19302100	900	HYPO	80	79.9	92	86.9	9.55	<0.001	Vmn2r98	82926	NM_001104550
chr17	21145401	21146300	900	HYPO	6	6.2	8.3	80	16.6	<0.001	Zfp160	0	NM_145483
chr17	27177001	27178500	1500	HYPO	35.6	37.3	51.1	45.9	12.05	<0.001	Ggnbp1	3678	NM_027544
chr17	27441801	27442700	900	HYPO	75.5	73.4	82.5	87.6	10.65	<0.001	.	.	.
chr17	40820401	40821300	900	HYPO	69.5	69.1	87.7	87.9	18.5	<0.001	Gm6084	-42768	NM_001038500
chr17	47640801	47641700	900	HYPO	47.5	50	60	73.1	22.75	<0.001	Ccnd3	-299	NM_001081635
chr17	50391801	50392700	900	HYPO	73.8	84.8	93.3	100	17.4	<0.001	Dazl	26018	NM_010021

chr17	55347601	55348700	1100	HYPO	67.6	67.7	90.5	84.7	19.85	<0.001	.	.	.
chr17	61669201	61670100	900	HYPO	47.9	43.9	61.2	54.1	11.7	<0.001	.	.	.
chr17	88996601	88997700	1100	HYPO	14.3	13.9	24.6	25.7	11.1	<0.001	Klraq1	8894	NM_028658
chr18	43846801	43847700	900	HYPO	18.6	18	27.6	22	10	<0.001	Jakmip2	0	NM_001163637
chr18	57722401	57723300	900	HYPO	36.8	31.5	47.2	47.9	12.85	<0.001	1700011I03Rik	0	NM_029290
chr18	65071001	65071900	900	HYPO	48.9	45.2	56.8	56	11.1	<0.001	Nedd4l	0	NM_001114386
chr18	76771201	76772700	1500	HYPO	18.5	9.2	20.1	27.2	12.05	<0.001	.	.	.
chr19	12288201	12288900	700	HYPO	18.7	29.4	40	37.7	14.85	<0.001	Olfr1431	3205	NM_146414
chr19	25679201	25680300	1100	HYPO	4.8	0	22.4	7.2	17.3	<0.001	Dmrt1	383	NM_015826
chr19	48306001	48306900	900	HYPO	63.4	74.5	90.6	89.1	20.85	<0.001	Sorcs3	0	NM_025696
chrX	7663401	7664300	900	HYPO	19.3	29.2	40.7	44.5	18.35	<0.001	Was	0	NM_009515
chrX	10675801	10676500	700	HYPO	5	11.1	36.1	.	28.05	<0.001	.	.	.
chrX	11577601	11579100	1500	HYPO	13.4	13.2	23.4	26	11.4	<0.001	Bcor	34763	NM_029510
chrX	36994001	36994900	900	HYPO	59.9	59.8	68.6	73.3	11.15	<0.001	.	.	.
chrX	58665801	58666700	900	HYPO	62.2	65.4	80.1	74.2	13.4	<0.001	.	.	.
chrX	63783401	63784300	900	HYPO	18.2	14	28.6	.	12.5	<0.001	.	.	.
chrX	72036201	72036900	700	HYPO	8.8	16	28.1	27.9	15.55	<0.001	Gm5936	-45634	NM_001081670
chrX	115053601	115054500	900	HYPO	64.6	65.2	77.6	71.1	9.5	<0.001	.	.	.
chrX	122875801	122876700	900	HYPO	52	53.1	61.7	67.6	12.05	<0.001	.	.	.

Appendix E: Summary of significantly differentially methylated genes (p<0.001)

Brain DOWN	Brain UP	Liver DOWN	Liver UP	Placenta DOWN	Placenta UP
1700047I17Rik1	*Aebp2*	*Agmat*	*2310040C09Rik*	*Rapgef3*	*Gzmd*
1700108L22Rik	*BC016423*	*Arg1*	*6430530L21Rik*	*Unc45a*	
2010007L18Rik	*Cdca4*	*Atf5*	*Art4*	*Unc5b*	
2310036D22Rik	*Cwf19l2*	*Azgp1*	*BC038156*	*Wdfy1*	
2510003E04Rik	*Gli3*	*BC032265*	*Cbfa2t3h*		
2510008P16Rik	*Igf2bp2*	*Bhmt2*	*Cdca8*		
2700050C19Rik	*Mmp2*	*C8g*	*D930049F02Rik*		
2810453I06Rik	*Plagl2*	*Chi3l3*	*Emilin2*		
5730455O13Rik	*Sepn1*	*Chi3l4*	*Entpd4*		
5730494M16Rik	*Vim*	*Cml1*	*Hemgn*		
5730494N06Rik		*Cyp2d26*	*Kif4*		
6330419J24Rik		*D4Bwg0951e*	*LOC637353*		
9130024F11Rik		*E130112E08Rik*	*Mier3*		
Abcd3		*EG665378*	*Mmrn1*		
Abce1		*Gls2*	*Pcyt1b*		
AI851790		*Gsta3*	*Pip5k1b*		
Ambp		*Gsta3*	*Pklr*		
Apoa2		*Hao1*	*Polh*		
Apoa2		*LOC667337*	*Sfrs4*		
Atp6v1a		*Nalp6*	*Slc43a1*		
Bhlhb2		*Pon1*	*Smarcc1*		
Cd200		*Tff3*	*Ssx2ip*		
Cfl2		*Wdfy1*	*Thumpd1*		
Chmp2a			*Usp7*		
Cox7a2					
Cstf3					
Ctnna2					
Dcun1d5					
Dlx2					
Efha1					
Efnb2					
Eif4e					
Eno1					
Esd					
Faim					
Fam134a					
Gad1					
Gap43					
Gjd2					
Glrx3					
Grp					
Hnrpll					
Hsdl1					
Id2					
Ifit2					
Kng1					
LOC100040413					
LOC100040671					
LOC100042773					
LOC100043906					
LOC100044087					

LOC100044170
LOC100045776
LOC231368
LOC277856
LOC380665
LOC380707
LOC381230
LOC382096
LOC383125
LOC635470
LOC665281
LOC668038
LOC668239
LOC668837
LOC674611
LOC674706
Mdh1
Minpp1
Mrpl13
Mrpl20
Msl2l1
mt-Nd4l
Mtrf1l
Nap1l5
Napa
Ndufb5
Ndufb9
Nef3
Nicn1
Nkain2
Nt5c
Nudcd2
Osbpl2
Otud6b
Oxr1
Pdcd5
Pgk1
Pkig
Poldip3
Ppa2
Ppp2r2c
Prdx5
Prkcb
Rchy1
Rnasek
Robld3
Rpl18a
Rwdd4a
S100a8
S100a9
Scoc
Selk
Serpina1b
Serpina1b
Serpina1b
Smc5l1
Sp1

Stfa1
Stfa1
Tceal1
Tcf7l2
Terf2ip
Tmed2
Tmem176b
Tmem55b
Tmem66
Tmem85
Tram1
Trf
Tuba3a
Tusc1
Twistnb
Txndc12
Uck1
Uhrf1bp1l
Vapa
Vhl
Vps26b
Vps36
Vstm2a
Wdfy1
Ythdf3
Zfand6
Zhx1

Down = downregulated in ethanol sired embryos, Up = upregulated in ethanol sired embryos compared to controls.

Appendix F: DNA Isolation from E16.5 Tails

Protocol adapted from *DNA Isolation from Ear Punches/Yolk Sac Tissue* protocol compliments of Dr Erica Watson (Centre for Trophoblastic Research, Department of Physiology, Development and Neuroscience, University of Cambridge, UK).

Table 21: Tail lysis buffer

Reagent	Quantity	Final Concentration
KCl (Sigma P951)	0.288g	50mM
1M Tris□HCl (pH 8.3) (Sigma)	0.5mL	10mM
1M $MgCl_2$ (Sigma)	0.1mL	2.0mM
Gelatin (Sigma)	5mg	0.1mg/mL
Tween-20 (Sigma)	225µL	0.45%
Nonidet P-40 (Roche)	225µL	0.45%

1. Make up to 50mL with ddH_2O
2. Autoclave
3. Add 0.25mL of 20mg/mL Proteinase K (Sigma) for a final concentration of 0.1mg/mL
4. Put the whole E16.5 tail into an 1.5mL microfuge tube and add 200µL of lysis buffer
5. Incubate in a shaking incubator at 55°C overnight at 600rpm
6. Heat at 94°C in heating block for 10-15 minutes to inactivate the residual Proteinase K
7. Spin again briefly
8. Dilute DNA samples 1:20 in ddH_2O
9. Use 1µL for PCR analysis (for a 25µL PCR reaction)

Appendix G: Combined functional annotation clustering of both brain and liver GO enrichment of overlap genes

Annotation Cluster 1	Enrichment Score: 1.930654403891769		
Category	Term	*p*-value	Genes
GOTERM_BP_FAT	GO:0008283~cell proliferation	7.91E-04	CCND3, TGFBR3, ID4, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0008284~positive regulation of cell proliferation	0.012451104	ODC1, ID4, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0042127~regulation of cell proliferation	0.013075856	ODC1, TGFBR3, ID4, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0045596~negative regulation of cell differentiation	0.039921273	TGFBR3, ID4, TGFB2
GOTERM_BP_FAT	GO:0040007~growth*	0.043209047	TGFBR3, BCL2L1, TGFB2

Annotation Cluster 2	Enrichment Score: 1.4294707811294596		
Category	Term	*p*-value	Genes
GOTERM_BP_FAT	GO:0007276~gamete generation	0.018472108	LMNA, CTCFL, BCL2L1, GGNBP1
GOTERM_BP_FAT	GO:0019953~sexual reproduction	0.027747724	LMNA, CTCFL, BCL2L1, GGNBP1
GOTERM_BP_FAT	GO:0048609~reproductive process in a multicellular organism	0.032335604	LMNA, CTCFL, BCL2L1, GGNBP1
GOTERM_BP_FAT	GO:0032504~multicellular organism reproduction	0.032335604	LMNA, CTCFL, BCL2L1, GGNBP1
GOTERM_BP_FAT	GO:0007283~spermatogenesis	0.07031286	LMNA, BCL2L1, GGNBP1
GOTERM_BP_FAT	GO:0048232~male gamete generation	0.07031286	LMNA, BCL2L1, GGNBP1

Annotation Cluster 3	Enrichment Score: 1.0766046308125428		
Category	Term	*p*-value	Genes
GOTERM_BP_FAT	GO:0006461~protein complex assembly	0.058249537	VWF, IGF1R, PSMG2
GOTERM_BP_FAT	GO:0070271~protein complex biogenesis	0.058249537	VWF, IGF1R, PSMG2
GOTERM_BP_FAT	GO:0065003~macromolecular complex assembly	0.112419287	VWF, IGF1R, PSMG2
GOTERM_BP_FAT	GO:0043933~macromolecular complex subunit organization	0.129465991	VWF, IGF1R, PSMG2

Annotation Cluster 4	Enrichment Score: 1.0339039185628365		
Category	Term	*p*-value	Genes
GOTERM_BP_FAT	GO:0043523~regulation of neuron apoptosis	0.008180538	GRM7, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0042981~regulation of apoptosis	0.069186771	PSMG2, GRM7, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0043067~regulation of programmed cell death	0.071343088	PSMG2, GRM7, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0010941~regulation of cell death	0.072276759	PSMG2, GRM7, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0006915~apoptosis	0.192164481	PSMG2, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0012501~programmed cell death	0.196067664	PSMG2, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0008219~cell death	0.217725266	PSMG2, BCL2L1, TGFB2
GOTERM_BP_FAT	GO:0016265~death	0.223679009	PSMG2, BCL2L1, TGFB2

Annotation Cluster 5	Enrichment Score: 0.6865005179585499		
Category	Term	*p*-value	Genes
GOTERM_CC_FAT	GO:0031967~organelle envelope	0.167442515	LMNA, BCL2L1, AKAP1
GOTERM_CC_FAT	GO:0031975~envelope	0.168453885	LMNA, BCL2L1, AKAP1
GOTERM_CC_FAT	GO:0031090~organelle membrane*	0.30913749	LMNA, BCL2L1, AKAP1

Annotation Cluster 6	Enrichment Score: 0.6616934341985168		
Category	Term	*p*-value	Genes
KEGG_PATHWAY	mmu04510:Focal adhesion	0.005612594	VWF, IGF1R, CCND3, ITGB5
SP_PIR_KEYWORDS	cleavage on pair of basic residues	0.060694828	VWF, IGF1R, TGFB2
GOTERM_BP_FAT	GO:0022610~biological adhesion	0.255663368	VWF, ITGB5, TGFB2
GOTERM_BP_FAT	GO:0007155~cell adhesion	0.255663368	VWF, ITGB5, TGFB2
SP_PIR_KEYWORDS	signal	0.381232489	VWF, IGF1R, GRM7, TGFBR3, ITGB5, SEZ6, TGFB2
UP_SEQ_FEATURE	signal peptide	0.479395865	VWF, IGF1R, GRM7, TGFBR3, ITGB5, SEZ6, TGFB2
SP_PIR_KEYWORDS	disulfide bond	0.611346906	VWF, IGF1R, ITGB5, SEZ6, TGFB2

UP_SEQ_FEATURE	disulfide bond	0.662635423	VWF, IGF1R, ITGB5, SEZ6, TGFB2
GOTERM_CC_FAT	GO:0044459~plasma membrane part	0.672450298	VWF, ITGB5, SEZ6

Annotation Cluster 7 Enrichment Score: 0.44194835397368576

Category	Term	*p*-value	Genes
GOTERM_CC_FAT	GO:0005886~plasma membrane	0.027847114	VWF, IGF1R, XPR1, GRM7, SLC2A2, TGFBR3, ITGB5, SEZ6, GGNBP1
GOTERM_BP_FAT	GO:0048729~tissue morphogenesis	0.062979111	IGF1R, TGFBR3, TGFB2
GOTERM_BP_FAT	GO:0007167~enzyme linked receptor protein signaling pathway	0.082077519	IGF1R, TGFBR3, TGFB2
UP_SEQ_FEATURE	topological domain:Extracellular	0.219026694	IGF1R, XPR1, GRM7, SLC2A2, TGFBR3, ITGB5, SEZ6
GOTERM_CC_FAT	GO:0044421~extracellular region part	0.285500398	VWF, TGFBR3, TGFB2
GOTERM_BP_FAT	GO:0006468~protein amino acid phosphorylation	0.304598816	IGF1R, TGFBR3, TGFB2
GOTERM_BP_FAT	GO:0016310~phosphorylation	0.353191246	IGF1R, TGFBR3, TGFB2
SP_PIR_KEYWORDS	signal	0.381232489	VWF, IGF1R, GRM7, TGFBR3, ITGB5, SEZ6, TGFB2
GOTERM_CC_FAT	GO:0005576~extracellular region	0.409772599	VWF, TGFBR3, SEZ6, TGFB2
SP_PIR_KEYWORDS	glycoprotein	0.416937006	VWF, IGF1R, GRM7, SLC2A2, TGFBR3, ITGB5, SEZ6, TGFB2
GOTERM_BP_FAT	GO:0007166~cell surface receptor linked signal transduction	0.422850882	IGF1R, GRM7, BRD7, TGFBR3, ITGB5, TGFB2
UP_SEQ_FEATURE	topological domain:Cytoplasmic	0.429049858	IGF1R, XPR1, GRM7, SLC2A2, TGFBR3, ITGB5, SEZ6
SP_PIR_KEYWORDS	Secreted	0.439870661	VWF, TGFBR3, SEZ6, TGFB2
GOTERM_BP_FAT	GO:0006793~phosphorus metabolic process	0.446980334	IGF1R, TGFBR3, TGFB2
GOTERM_BP_FAT	GO:0006796~phosphate metabolic process	0.446980334	IGF1R, TGFBR3, TGFB2
UP_SEQ_FEATURE	signal peptide	0.479395865	VWF, IGF1R, GRM7, TGFBR3, ITGB5, SEZ6, TGFB2
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	0.480388322	VWF, IGF1R, GRM7, SLC2A2, TGFBR3, ITGB5, SEZ6, TGFB2
GOTERM_CC_FAT	GO:0016021~integral to membrane	0.550727957	IGF1R, XPR1, GRM7, SLC2A2, TGFBR3, ITGB5, BCL2L1, AKAP1, SEZ6
SP_PIR_KEYWORDS	cell membrane	0.569660605	XPR1, GRM7, TGFBR3, SEZ6
GOTERM_CC_FAT	GO:0031224~intrinsic to membrane	0.604719715	IGF1R, XPR1, GRM7, SLC2A2, TGFBR3, ITGB5, BCL2L1, AKAP1, SEZ6
SP_PIR_KEYWORDS	receptor	0.604980959	IGF1R, XPR1, GRM7, TGFBR3, ITGB5
SP_PIR_KEYWORDS	disulfide bond	0.611346906	VWF, IGF1R, ITGB5, SEZ6, TGFB2

SP_PIR_KEYWORDS	membrane	0.624224636	IGF1R, XPR1, GRM7, SLC2A2, TGFBR3, ITGB5, BCL2L1, AKAP1, SEZ6, GGNBP1
UP_SEQ_FEATURE	disulfide bond	0.662635423	VWF, IGF1R, ITGB5, SEZ6, TGFB2
UP_SEQ_FEATURE	transmembrane region	0.687024567	IGF1R, XPR1, GRM7, SLC2A2, TGFBR3, ITGB5, BCL2L1, SEZ6
SP_PIR_KEYWORDS	transmembrane	0.699662264	IGF1R, XPR1, GRM7, SLC2A2, TGFBR3, ITGB5, BCL2L1, AKAP1, SEZ6

Annotation Cluster 8	Enrichment Score: 0.3502782264286508		
Category	Term	*p*-value	Genes
GOTERM_BP_FAT	GO:0045449~regulation of transcription	0.167353463	BRD7, CTCFL, TGFBR3, ID4, CDC5L, BCOR, ZFP317
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-dependent	0.724689066	BRD7, BCOR, ZFP317
GOTERM_BP_FAT	GO:0051252~regulation of RNA metabolic process	0.73346418	BRD7, BCOR, ZFP317

Annotation Cluster 9	Enrichment Score: 0.33765320278442934		
Category	Term	*p*-value	Genes
GOTERM_BP_FAT	GO:0045449~regulation of transcription	0.167353463	BRD7, CTCFL, TGFBR3, ID4, CDC5L, BCOR, ZFP317
SP_PIR_KEYWORDS	Transcription	0.584326224	CTCFL, CDC5L, BCOR, ZFP317
GOTERM_BP_FAT	GO:0006350~transcription	0.603294273	CTCFL, CDC5L, BCOR, ZFP317
SP_PIR_KEYWORDS	transcription regulation	0.756080272	CTCFL, CDC5L, BCOR
Annotation Cluster 10	Enrichment Score: 0.03085137506006925		
Category	Term	*p*-value	Genes
SP_PIR_KEYWORDS	zinc	0.840071803	RNF123, CTCFL, ZFP317
SP_PIR_KEYWORDS	metal-binding	0.849158736	IGF1R, RNF123, CTCFL, ZFP317
GOTERM_MF_FAT	GO:0008270~zinc ion binding	0.899942194	RNF123, CTCFL, ZFP317
GOTERM_MF_FAT	GO:0046914~transition metal ion binding	0.956930712	RNF123, CTCFL, ZFP317
GOTERM_MF_FAT	GO:0046872~metal ion binding	0.996372251	RNF123, CTCFL, ZFP317
GOTERM_MF_FAT	GO:0043169~cation binding	0.996630241	RNF123, CTCFL, ZFP317
GOTERM_MF_FAT	GO:0043167~ion binding	0.996975056	RNF123, CTCFL, ZFP317